Process development of lentiviral vector expression, purification and formulation for gene therapy applications

Thesis submitted for the degree of

Doctor of Philosophy

by

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To my father.

Tack för allt.

Declaration

I, Sara Margareta Nilsson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature

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ABSTRACT

There is growing interest in the use of lentiviral vectors, particularly for cancer immunotherapy and the treatment of monogenic diseases. Manufacturing of these vectors is challenging primarily due to cytotoxic effects of vector components resulting in low cell culture titres and vector instability leading to low purification yields. In addition, currently used processes are typically not scalable as they rely on adherently cultured cells and unit operations such as batch centrifugation and gel filtration.

To improve process scalability, suspension adaptation of a lentiviral vector packaging cell line was attempted, however, cell aggregation could not be prevented. For vector clarification it was found that membranes with pore sizes of 0.22 μm resulted in recoveries less than 50%, whereas the use of 0.45 μm membranes resulted in recoveries close to 100%. Successful vector concentration utilising centrifugal filters was possible with a membrane molecular weight cut-off (MWCO) of 100 kDa, whereas a 300 kDa MWCO led to low recoveries.

Chromatography stationary phases that allow convective mass transfer, such as membranes and monoliths, are becoming increasingly popular for purification of large molecules. Lentiviral vector was found to bind monoliths with weak and strong anion exchangers over a wide range of conditions. Vector elution conditions determined for membrane- and monolith-based resins in high-throughput 96-well plate format were found to not be indicative of gradient elution conditions for 1 mL versions of these resins operated by a chromatography system.

The thermolability of lentiviral vectors leads to a requirement for storage at less than -65°C. Inexpensive mixtures of sugars in combination with a glycine-derivative were studied for their ability to stabilise a lentiviral vector during freeze-drying and subsequent thermochallenge. The amorphous solid formed upon freeze-drying was able to stabilise the vector for up to 12 weeks at 4°C and eight weeks at 25°C. This is in comparison to formulation in phosphate buffered saline, where more than 90% of infectious titre was lost immediately upon freeze-drying.

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ABBREVIATIONS AND SYMBOLS

Abbreviation	Description
AAVs	Adeno-associated vectors
ADA-SCID	Adenosine deaminase deficiency-SCID
AEX	Anion exchange
BCA	Bichinchonic acid
BIV	Bovine immunodeficiency virus
BSA	Bovine serum albumin
BV	Baculovirus
CaPi	Calcium phosphate
CAR	Chimeric antigen receptor
CCF	Cell culture fluid
CCS	Cell culture supernatant
CF	Concentration factor
CHO	Chinese hamster ovary
CIM	Convective Interaction Media
CMC	Chemistry, Manufacturing and Controls
CMO	Contract manufacturing organisation
COGs	Cost of goods
CP	Centre point
CVs	Column volumes
DBC	Dynamic binding capacity
DCs	Dendritic cells
DEAE	Diethylaminoethyl
DF	Diafiltration
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
DoE	Design of experiment
dox	Doxycycline
dsDNA	Double stranded DNA
EBA	Expanded bed adsorption

Abbreviation	Description
eGFP	Enhanced green fluorescent protein
EIAV	Equine infectious anaemia virus
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
eVLPs	Enveloped virus-like particles
FBS	Fetal bovine serum
FFF-MALLS	Field-flow fractionation multiple-angle laser light scattering
FIV	Feline immunodeficiency virus
GMP	Good manufacturing practice
GSK	GlaxoSmithKline
GTA	Gene transfer assay
HBSS	Hanks' Balanced Salt Solution
HCPs	Host cell proteins
HEK 293T	Human embryonic kidney 293T
HIV-1	Human immunodeficiency virus type 1
HSA	Human serum albumin
IMAC	Immobilised metal affinity chromatography
LTRs	Long terminal repeats
LV	Lentiviral vector
MAA	Marketing authorisation application
mAbs	Monoclonal antibodies
MLD	Metachromatic leukodystrophy
MOI	Multiplicity of infection
MoMLV	Moloney murine leukaemia virus
MWCO	Molecular weight cut-off
NTA	Nanoparticle tracking analysis
OFAT	One-factor-at-a-time
PBS	Phosphate buffered saline
pDNA	Plasmid DNA
PEI	Polyethylenimine
PES	Polyethersulfone
PVDF	Polyvinylidene fluoride

Abbreviation	Description
Q	Quaternary ammonium
QbD	Quality by design
qPCR	Quantitative polymerase chain reaction
RCL	Replication-competent lentivirus
RSD	Relative standard deviation
SCID-X1	X-linked severe combined immunodeficiency
SEC	Size exclusion chromatography
SFDA	State Food and Drug Administration
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
ssRNA	Single-stranded RNA
STR	Stirred tank bioreactor
SU	Surface subunit
TCR	T cell receptor
TE	Tris-EDTA
tet	Tetracycline
TM	Membrane spanning subunit
TRE	Tetracycline response element
TRPS	Tunable resistive pulse sensing
TSE	Transmissible spongiform encephalopathies
TU	Transducing units
UF	Ultrafiltration
USD	Ultra-scale down
VSV-G	Vesicular stomatitis virus G protein
WAS	Wiskott-Aldrich syndrome
WPRE	Posttranscriptional regulatory element of woodchuck hepatitis virus

Symbol	Description	Units
t	Filtration time	min
V	Cumulative filtrate volume	mL
Q_0	Initial filtrate flow rate	mL min^{-1}
V_{max}	Maximum volume that can be filtered before membrane becomes completely plugged by foulant	mL
q	Protein binding capacity	mg mL^{-1}
$m_{protein,\ loaded}$	Mass of protein added to resin during load step	mg
$m_{protein,\ remaining}$	Mass of protein found in flow-through fraction	mg
V_{resin}	Volume of chromatography resin	mL

1. INTRODUCTION

1.1 Thesis overview

This thesis examines some of the challenges associated with the manufacturing of lentiviral vectors (LVs) for application to human gene therapy. The thesis will have the following structure:

Chapter 1 presents a review of the literature for gene therapy, with an emphasis on LVs and their manufacturing.

Chapter 2 describes suspension adaptation, characterisation and development of cell culture, harvest and concentration steps for a LV based on human immunodeficiency virus type 1 (HIV-1).

Chapter 3 covers chromatographic purification of a HIV-1-based LV using membrane-based and monolithic stationary phases.

Chapter 4 provides a study of stability and formulation in lyophilised and liquid formats of a HIV-1-based LV.

Chapter 5 offers concluding remarks and ideas for future work.

1.2 Gene therapy

Gene therapy involves the treatment of a disease or medical disorder by the introduction of therapeutic genetic information into the appropriate cellular targets (Escors and Breckpot, 2012). The therapeutic genetic information could, for example, encode for a faulty gene, as in the case in gene therapy for adenosine deaminase deficiency-severe combined immunodeficiency (ADA-SCID), or for a cancer antigen receptor as with chimeric antigen receptor (CAR) T cell therapy for treatment of acute lymphoblastic leukaemia (ALL). After decades of research, the beginning of this century saw the first successful application of gene therapy in humans in the form of clinical trials to cure X-linked SCID (SCID-X1) utilising a γ-retroviral vector (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002). However, the success was overshadowed by the

development of vector-related leukaemia in a few of the patients (Hacein-Bey-Abina et al., 2003). This, in combination with the death of an American teenager on a clinical trial due to an inflammatory reaction to the adenoviral vector used (Raper et al., 2003), halted further clinical progress until vector safety was improved. Perhaps because of a lack of fatal adverse events in trials in the region, in 2003, the Chinese State Food and Drug Administration (SFDA) made China the first country to approve the commercial production of a gene therapy product (Pearson et al., 2004). The treatment – marketed as Gendicine – targets head and neck squamous cell carcinoma and consists of an adenoviral vector encoding p53 tumour suppressor gene in combination with radiotherapy (Peng, 2005). In 2005, Oncorine, another adenoviral vector-based product, became the second gene therapy to gain marketing authorisation in China (Ledley et al., 2014). These two treatments were followed in 2011 by the decision of the Russian Ministry of Healthcare and Social Development to grant approval for a naked plasmid DNA (pDNA) gene therapy for the treatment of peripheral arterial disease (marketed with the trade name Neovasculogen). Progress in the EU and the US has been slower, but in 2012 the European Medicines Agency (EMA) granted a Dutch biotech company marketing authorisation for their adeno-associated vector-based treatment for lipoprotein lipase deficiency (EMA, 2012). The high cost of the treatment (close to $1 million per patient) has been the source of much discussion (Morrison, 2015). However, health economics and the value-based pricing system are likely to support high prices for one-time, potentially curative treatments for lifetime illnesses (Brennan and Wilson, 2014). The reimbursement strategies for cell and gene therapies may need to be altered compared to those for traditional therapies, e.g., payment in stages based on clinical outcome (Abou-El-Enein et al., 2014), but parallels with reimbursement for many vaccines may provide helpful. This will likely provide an array of challenges across regions with different healthcare payment models. Recently, GlaxoSmithKline (GSK) have filed a marketing authorisation application (MAA) to the EMA for a γ-retroviral vector-based *ex vivo* therapy for treatment of ADA-SCID (GSK, 2015). This marks the entry of big pharma into the previously considered unattractive cell and gene therapy area. A review by Seymour and Thrasher (2012) highlights some of the most promising clinical studies carried out to date. As of June 2014, more than 2,000 gene therapy clinical trials have been undertaken (Gene therapy clinical trials worldwide, 2014). Viral vectors have been the most commonly used method of gene delivery (Gene therapy clinical trials worldwide, 2014), but safety concerns related to these vectors

have led to increased use of non-viral vectors, such as naked DNA and lipofection. However, non-viral vectors are less efficient and long-lasting transgene expression typically cannot be achieved (Glover et al., 2005; Gillett et al., 2009).

1.3 Viral vectors for gene therapy

No single vector is likely to suit all gene therapy applications, as described below, therefore a number of virus types are being investigated for use as gene delivery vectors, including adenoviruses, retroviruses and vaccinia virus (Gene therapy clinical trials worldwide, 2014). In clinical trials, adenoviruses have been the most frequently used, followed closely by γ-retroviruses, while other viral vectors have only been used rarely.

Adenoviruses are non-enveloped viruses with double stranded DNA genomes. There are over 50 adenoviral serotypes, with types 2 and 5 most commonly used as gene therapy vectors as they are the serotypes most adults have been exposed to (Kay et al., 2001). Prior exposure to these serotypes means that pre-existing immunity complicates the use of these vectors, and the death of a teenager enrolled in a clinical trial employing an adenoviral vector was the result of an immune response (Somia and Verma, 2000). Adenoviral vectors were developed to suit *in vivo* applications and as genomic integration of the vector is not possible with this vector type, they are best suited for applications where the target cells are non-replicating or where transient gene expression is sufficient. The key advantages of adenoviral vectors are their high efficiency of transduction and high level of gene expression, however, expression is transient and declines rapidly (Edelstein et al., 2007; Somia and Verma, 2000). Further advantages include the capacity to infect non-replicating cells and the ability to deliver larger genetic loads than γ-retroviral vectors. The main disadvantages relate to safety and in particular the possibility of provoking a severe immune and inflammatory response. Many of the disadvantages of adenoviral vectors, such as transgene silencing and immunogenicity, are being overcome by the use of second-generation adeno-associated vectors (AAVs) (Wilson, 2011).

γ-retroviruses, such as Moloney murine leukaemia virus (MoMLV), were the first vectors developed and are suitable for *ex vivo* therapy approaches where long-term gene expression is required. Long-term transgene expression is possible due to the ability of retroviruses to integrate into the host-cell genome. The use of γ-retrovirus-based gene

delivery vectors was proposed in the 1980s by Mann et al. γ-retroviral vectors became popular mainly because of their aforementioned capability to integrate their genome into the host-cell chromosomes, thus providing stable gene transfer (Edelstein et al., 2007). However, this property has also been their biggest drawback, as demonstrated by the leukaemia-like condition observed in the SCID-X1 trial mentioned in Section 1.2. In addition to issues with genotoxicity, γ-retroviral vectors require mitotic cells for transduction, which complicates their use for cell types such as neurons and stem cells.

The *Retroviridae* family consists of seven different genera: the α-, β-, γ-, δ- and ε-retroviruses, as well as the spumaviruses and lentiviruses (Gillet et al., 2009). Based on their genome organisation, retroviruses are divided into simple and complex retroviruses (Escors and Breckpot, 2010). Simple retroviruses, such as γ-retroviruses, have been used more commonly as gene therapy vectors than complex retroviruses, such as lentiviruses. LVs, however, can transduce quiescent cells and exhibit reduced insertional mutagenesis. These properties have led to LVs becoming increasingly popular for use in research and clinical trials.

1.3.1 Lentiviral vectors

LVs, like γ-retroviral vectors, have the ability to stably integrate into the target cell genome, thus allowing persistent expression of the gene of interest. However, unlike γ-retroviral vectors, LVs can also transduce postmitotic cells and accommodate larger transgenes (Lewis et al., 1992; Mátrai et al., 2010). Many target cells, such as neurons, haematopoietic stem cells and dendritic cells (DCs), divide infrequently *in vivo* (Naldini et al., 1996; Collins and Cerundolo, 2004). The ability of LVs to transduce quiescent and postmitotic cells makes them very useful for a large number of gene therapy applications. It should be pointed out that it is an oversimplification to state that LVs do not require dividing cells for transduction, as they still require target cells to be stimulated to progress into the late G1 phase of the cell cycle for efficient transduction, however, transduction efficiency is significantly improved compared to γ-retroviral vectors (Naldini, 2011). In addition, self-inactivating (SIN) (described in Section 1.5) LVs appear to have reduced genotoxicity compared to γ-retroviral vectors, possibly due to their different integration profile, which indicates that LV integration is less likely to lead to oncogenesis (Mátrai et al., 2010). LVs also have a safety advantage over

adenoviral vectors due to the lack of innate and cellular responses against vector associated proteins (Abordo-Adesida et al., 2005).

The extensive body of work on HIV-1 has led to it becoming the lentivirus most commonly reengineered and used as a gene therapy vector (Lever et al., 2004). However, other lentiviruses such as HIV-2, equine infectious anaemia virus (EIAV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and bovine immunodeficiency virus (BIV) are all being explored for their potential as safe and efficient vectors. Nonprimate vectors have been considered safer than primate vectors as they are deemed less likely to become pathogenic if a replication-competent lentivirus (RCL) is created (Binder and Dropulic, 2008). Current vector design (discussed in Section 1.5) makes the possibility of an RCL remote and the safety distinctions between nonprimate and primate vectors may no longer be relevant.

In 1996 Naldini et al. reported the first successful laboratory application of LVs for gene delivery. The first clinical trial was initiated only seven years later and was aimed at treatment of HIV (Humeau et al., 2004). This initial trial was followed by a number of HIV trials (http://www.wiley.com/legacy/wileychi/genmed/clinical/). The safety database generated from these early studies helped clinical application of LVs for non-HIV conditions such as Wiskott-Aldrich syndrome (WAS), β-thalassemia and haematologic cancers. In the area of monogenic diseases, clinical trial results for *ex vivo* haematopoietic stem cell gene therapy for WAS (Aiuti et al., 2013), metachromatic leukodystrophy (MLD) (Biffi et al., 2013) and β-thalassaemia (Cavazzana-Calvo et al., 2010) have been very promising. The confidence in these results is illustrated by the fact that both small biotech companies, such as Bluebird Bio, and pharmaceutical multinationals, including GSK and Biogen, are working to commercialise these types of therapies. The same is also true of application of LVs to treat haematologic cancers, where biotech companies, such as Juno Therapeutics, are joined by giants Novartis, Johnson & Johnson and Pfizer in attempting to bring chimeric antigen receptor (CAR) and T cell receptor (TCR) therapies to market (Hagen, 2014).

1.4 Lentivirus biology

Retroviruses, including the lentivirus subfamily, have single-stranded RNA (ssRNA) genomes of 7-11 kilobases, two of which homodimerise and package in lipid-enveloped viral particles (Kay et al., 2001). The spherical particles have an average mass of ~2.5 ×

10^5 kDa (Vogt and Simon, 1999) and measure ~80-120 nm in diameter (Segura et al., 2006).

Figure 1.1 illustrates a typical lentiviral particle and the main virus components. The two RNA copies, the enzymes reverse transcriptase, integrase and protease are all complexed with nucleocapsid protein (Escors and Breckpot, 2010). A second protein shell, formed by the capsid protein, encompasses the nucleocapsid. The viral core is surrounded by matrix proteins, which interact with the host cell-derived lipid envelope. The envelope contains viral envelope glycoproteins, comprising a transmembrane subunit, which anchors the protein into the lipid bilayer, and a surface subunit, which binds to cellular receptors. The overall composition of retroviral particles is 60-70% protein, 30-40% lipid, 2-4% carbohydrate and 1-2% RNA (Andreadis et al., 1999).

The genetic organisation of a generalised retroviral provirus is shown in Figure 1.2. The provirus can be broadly divided into complete genes and sequences that have other roles (Vogt, 1997). The three essential retroviral genes are *gag*, which encodes viral structural proteins; *pol*, which encodes the enzymes that accompany the ssRNA; and *env*, which encodes viral envelope glycoproteins (Somia and Verma, 2000). The viral genes are bracketed by the long terminal repeats (LTRs), which are identical sequences that can be divided into three parts: U3 stems from a sequence unique to the 3' end of the RNA; R is derived from a sequence repeated at both ends of the RNA; and U5 is derived from a sequence unique to the 5' end of the RNA (Vogt, 1997). U3 contains the majority of the control elements of the provirus, including the promoter and several enhancer elements responsive to cellular and in some cases viral transcriptional activator proteins.

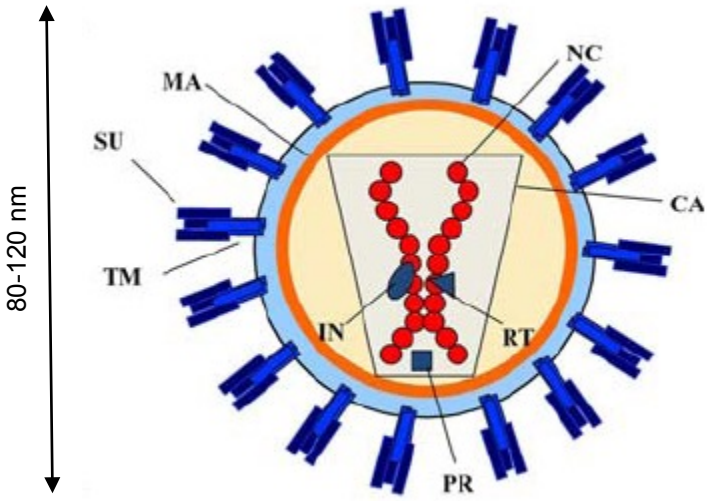


Figure 1.1 Schematic of LV showing the main vector components. Abbreviations: CA capsid, IN integrase, MA matrix, NC nucleocapsid, PR protease, RT reverse transcriptase, SU surface, TM transmembrane. Image from Escors and Breckpot, 2010.

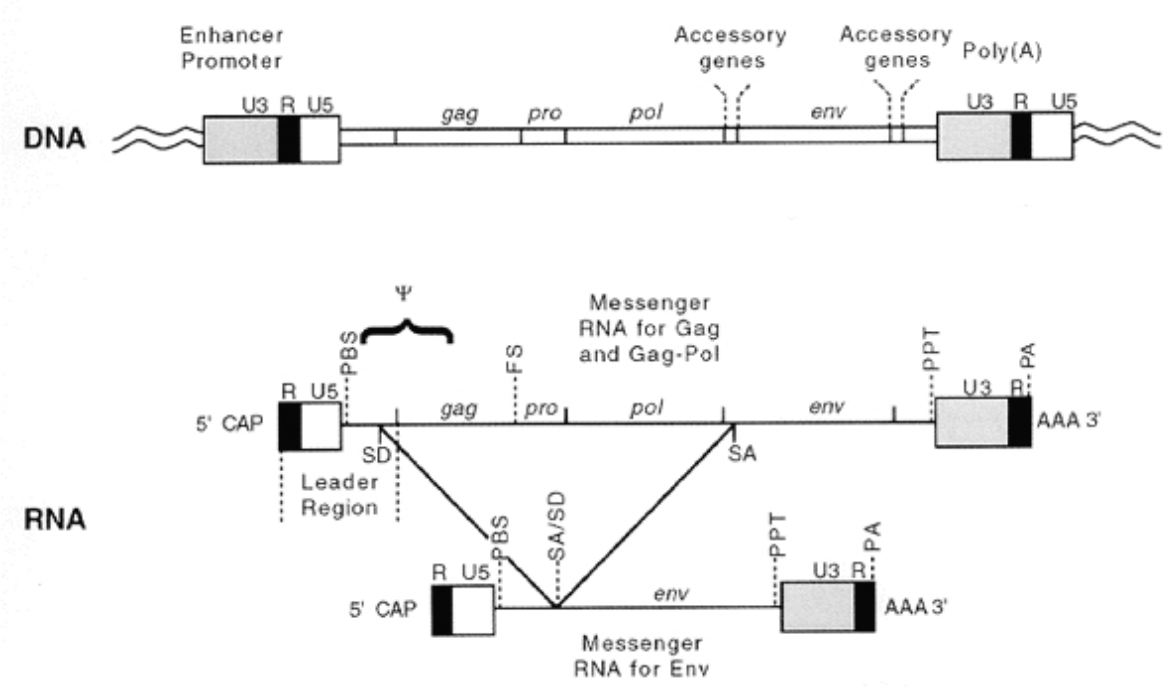


Figure 1.2 Genetic organisation of generalised retroviral provirus. The top line shows the proviral DNA as it is inserted into the host genome. The primary transcriptional product is shown on the second line, and the third line is the spliced messenger RNA for the Env protein. Abbreviations: PBS primer-binding site, ψ packaging site, SD splice donor site, FS frameshift site, SA splice acceptor site, PPT polypurine tract, PA polyadenylation signal, AAA poly(A) tail. Image from Vogt, 1997.

In relation to retroviral vectors (including LVs), the polyproteins Gag, Pol and Rev are required in *trans* for viral replication and packaging, whereas the 5' and 3' LTRs, the packaging site (ψ), the transport RNA-binding site, the integration sequences and additional sequences involved in reverse transcription are required in *cis* (Breckpot et al., 2007).

Lentiviruses have additional regulatory and accessory genes which allow them to control their gene expression. This also enables manipulation of the host cell both for virus production and for virus entry and integration of their genetic material into the cell genome (Lever et al., 2004). The two regulatory genes *tat* and *rev* act at the transcriptional and posttranscriptional level, respectively (Farson et al., 2001). The Tat protein promotes transcriptional elongation, whereas the Rev protein facilitates cytoplasmic export of unspliced and singularly spliced viral transcripts that express late viral proteins. Primate immunodeficiency viruses also carry the accessory genes *nef*, *vif*, and *vpr* (Cullen, 1998). In addition to these, HIV-1 also has the *vpu* accessory gene. The Nef protein has been shown to have at least three distinct roles in infected cells: down-regulation of cell surface CD4 expression; down-regulation of cell surface major histocompatibility complex I (MHC I); and enhanced virion infectivity. Vif is known to enhance the infectivity of HIV-1 virions produced in primary T cells, whereas Vpr plays a role in nuclear import of the preintegration complex and induces arrest in the G2 phase of the cell cycle. G2-arrested cells have been shown to have a more active HIV-1 LTR promoter, which may provide a rationale for this function of the Vpr protein. The function of Vpu is to enhance virion release from infected cells and to selectively degrade CD4 in the cell endoplasmic reticulum.

Figure 1.3 shows the lifecycle of a non-replication competent LV. This is largely similar to the lifecycle of a wild-type lentivirus, however, the genes expressed differ and hence LV transduction does not result in virus budding in the final step of the lifecycle. The first step in lentivirus infection is virus Env binding to receptors on the target cell (Figure 1.3 (1)) (Bukrinsky, 2014). This eventually results in fusion of the virus membrane to the target cell membrane and as capsid protein is dissociated a viral nucleoprotein complex is formed. The viral reverse transcriptase then catalyses the conversion of the two strands of viral RNA into a copy of double stranded DNA (dsDNA) (Figure 1.3 (2)). Upon completion of reverse transcription, the nucleoprotein complex, now referred to as the pre-integration complex, is translocated into the nucleus (Figure 1.3 (3)). This is in contrast to γ-retroviruses and γ-retroviral vectors, which

cannot traverse the nuclear membrane and therefore require mitotic cells for successful infection/transduction. Integrase proceeds to create a nick in the host cell chromosome, allowing the proviral DNA to integrate (Figure 1.3 (4)). It appears that the reduced genotoxicity of LVs compared to γ-retroviral vectors results from the fact that LVs preferentially integrate in transcriptionally active sites, whereas γ-retroviral vectors tend to integrate in transcription start sites (Cattoglio et al., 2010). It has been recently shown that HIV preferentially integrates in the outer shell of the nucleus in close correspondence with the nuclear pore (Marini et al., 2015). Increasing knowledge of HIV integration will hopefully facilitate increasingly safe LVs to be designed. Following integration, the viral genes (or transgene(s) for LVs) are transcribed and spliced (Figure 1.3 (5)). Viral genomic RNA and mRNA are transported to the cytoplasm where mRNA is translated. For LVs, this step corresponds to expression of the transgene(s) (Figure 1.3 (6)). Viral proteins and RNA then translocate to the assembly site where a virion is formed, matured and finally buds from the host cell membrane.

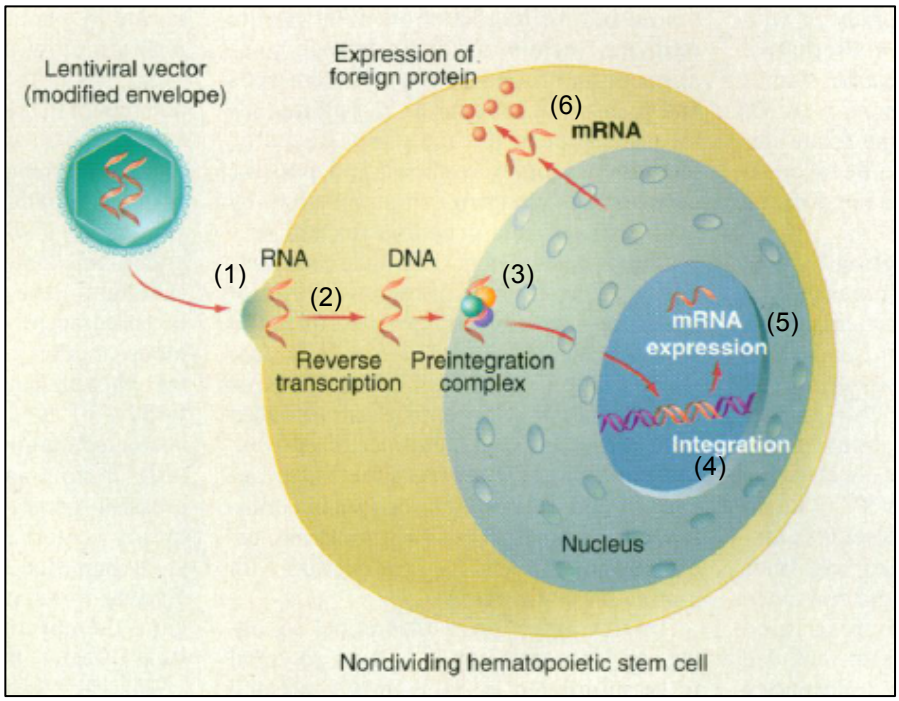


Figure 1.3 **Lentiviral vector lifecycle.** Image from Amado and Chen, 1999.

1.5 Lentiviral vector development

Vector safety is a key issue for vector design and, considering the pathogenicity of the parental virus, in the case of HIV-1-based vectors, the biosafety concerns are heightened. LV particles have traditionally been generated by the co-transfection of three plasmids in human embryonic kidney 293T cells (HEK 293T) (Naldini et al., 1996). The common method of separating constructs to three plasmids is used to reduce the possibility of recombination to replication-competent virus (Binder and Dropulic, 2008). The three plasmids comprise a packaging plasmid, a transfer plasmid and an envelope-encoding plasmid.

The design of HIV-1-based vectors has progressed through a number of so called generations (Escors and Breckpot, 2010). Included in the first-generation packaging plasmid were all *gag* and *pol* sequences, the viral regulatory genes *tat* and *rev*, as well as the accessory genes *vif*, *vpr*, *vpu* and *nef*. Through the identification of genes unnecessary for transfer of the therapeutic gene(s), second-generation packaging systems were developed where *vif*, *vpr*, *vpu* and *nef* were removed (Zufferey et al., 1997). This greatly improved the safety profile of LVs since any RCL would be devoid of all virulence factors. In third-generation packaging systems the *rev* gene is placed on an individual plasmid and a gene sequence on the transfer plasmid is replaced by a strong *tat*-independent constitutively active promoter sequence (Dull et al., 1998). *Tat* is essential for viral replication, thus by its removal the biosafety of LVs is further improved.

The transfer plasmid codes for the transgene, which is flanked by HIV-1 *cis*-acting sequences required for encapsidation, reverse transcription and integration (Farson et al., 2001). Modifications of the transfer plasmid are commonly carried out to improve vector safety and performance. One such modification is the addition of the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE), which has been shown to increase the expression of transgenes, presumably by affecting posttranscriptional processing and the nuclear export rate of mRNA (Zufferey et al., 1999). Another change to the transfer plasmid led to the development of SIN vectors (Miyoshi et al., 1998; Zufferey et al., 1998). A deletion in the 3' LTR, which, during reverse transcription, is transferred to the 5' LTR of the proviral DNA, results in the abolishment of LTR promoter activity and thus production of full-length vector RNA in transduced cells is not possible. In the presence of an RCL, e.g., during wild-type HIV-

infection, traditional transfer vector could be packaged and spread, however, when a SIN vector is used, the production of full-length vector RNA is abolished and consequently the vector cannot be mobilised.

The risk of oncogene activation due to the insertion of LV genetic material has led to interest in the development of non-integrating LVs (Escors and Breckpot, 2010). Mutations in the integrase-coding region of the packaging plasmid are used to eliminate integrase activity without affecting reverse transcription and transport of the pre-integration complex to the nucleus. This type of vector would be useful for applications where non-dividing cells are targeted as they would retain stable gene expression. Another potential application is vaccination, as long-term expression is not required. Recently, non-integrating LVs have been shown to be comparable to standard LVs in a tumour therapy model (Karwacz et al., 2009).

Alterations to promoters have been carried out to reduce gene silencing, to allow for tissue-specific gene expression or to permit inducible gene expression (Binder and Dropulic, 2008). Cell-specific gene expression ensures therapeutic effects in the desired cells while limiting side effects caused by gene expression in nontarget cells (Liu et al., 2004). The other advantage of cell-specific promoters is that they are less likely to activate the host-cell defence machinery, and they are therefore less sensitive to promoter inactivation. This leads to an expected improvement in the stability and longevity of gene expression. The regulation of transgene expression is desirable for several gene therapy applications, for example genetic diseases where expression levels and timing are important (Escors and Breckpot, 2010). Among the most widely researched inducible promoters are those based on the tetracycline (tet) system. For clinical application, vectors dependent on the delivery of antibiotic (tet-on) are preferable to vectors dependent on gene silencing (tet-off), as with the tet-off system, constant administration of antibiotic is required when transgene expression is not needed.

1.5.1 Viral envelope protein alteration

LV tropism is determined by the viral envelope glycoproteins, as it is the glycoproteins that interact with receptors on the cell of interest (Cronin et al., 2005). In a process known as pseudotyping, wild-type lentivirus envelopes are substituted for envelopes derived from other enveloped viruses. This is advantageous as wild-type lentivirus

envelopes have restricted tropism and do not allow production of high-titre LV preparations.

The most commonly used envelope for pseudotyping HIV-1-based vectors is the rhabdovirus vesicular stomatitis virus G protein (VSV-G). The use of VSV-G has two main advantages: firstly, it confers a broad host-cell range and secondly, it increases vector stability, thus allowing vector concentration using ultracentrifugation (Burns et al., 1993). The most significant disadvantage of VSV-G is that it is cytotoxic in all cell lines if constitutively expressed (Cronin et al., 2005). Cytotoxicity results from the fusogenic activity of VSV-G, causing syncytia to form (e.g., Kafri et al., 1999). However, regulatable promoters can be used to alleviate this problem. Another limitation of VSV-G pseudotypes is that they are inactivated by human serum complement (DePolo et al., 2000). This drawback may be overcome as the use of polyethylene glycol-modified VSV-G vectors has been found to protect against inactivation while not significantly compromising transduction efficiency (Callahan et al., 2004). In addition, LV-based treatments are likely to be carried out *ex vivo* and inactivation by human serum complement is not an issue.

For *ex vivo* gene therapy, vectors with broad tropism can be used (Bouard et al., 2009). However, for *in vivo* therapy, off-target transduction will affect the safety and efficiency of gene delivery. Targeting of the tissue of interest thus becomes necessary and broad-tropism pseudotypes such as VSV-G may not be useful. An array of glycoproteins has been successfully used for LV pseudotyping and examples include glycoproteins from *Rhabdoviridae*, *Arenaviridae*, *Togaviridae*, *Filoviridae*, *Retroviridae* and *Baculoviridae* (Cronin et al., 2005). However, naturally occurring envelope proteins that are cell-type specific are not always possible to find and therefore may need to be genetically engineered for the desired cell target. One such example was demonstrated by Yang et al. (2008), who reported high targeting specificity of dendritic cells *in vivo* using Sindbis virus glycoprotein. Sindbis virus shows natural preference for DCs, but it was possible to further increase targeting efficiency by restricting its binding to commonly expressed heparan sulphate.

1.6 Lentiviral vector bioprocessing

Similarly to γ-retroviral vector production, LV bioprocessing is heavily influenced by lentivirus characteristics, such as their large size compared to other biologics, sensitivity

to inhibitors, particle complexity and instability (Andreadis et al., 1999). The most significant challenge for upstream processing is the low product titres, whereas the challenge for downstream processing is maintaining infectivity. Retroviral particles typically have a half-life in the region of 6-7 hours at 37°C. This has implications for bioreactor mode of operation and puts an emphasis on the development of downstream processes with short processing times.

Virus particle instability results partly from the fragility of Env. γ-retrovirus Env is composed of a trimer of heterodimers (Strang et al., 2004). The dimers consist of an extraviral surface subunit (SU) and a membrane spanning subunit (TM). In the mature protein, SU and TM are bonded via non-covalent interactions and a labile disulphide bond. It is the weakness of these interactions that lead to vector instability, as dissociation of SU and TM causes inactivation of the virus. Components present in the cell culture media, such as soluble Env, have been reported to act as viral inhibitors, thus effectively reducing functional titre (Slingsby et al., 2000). Host cell-derived proteoglycans and glycosaminoglycans have also been described as inhibitors of transduction (Segura et al., 2006). Carmo et al. (2009) showed that the reduction of LV infectivity at 37°C correlates with the loss of reverse transcriptase activity. The mechanisms of LV inactivation, and methods to mitigate inactivation, is clearly an area that requires more research.

LV manufacturing difficulties have led to a high cost of goods (COGs) and clinical trials have highlighted the elevated cost of vector production as one of the main challenges for successful clinical application of gene therapy (Waehler et al., 2007). The development of bioprocesses that produce LVs of appropriate quality in a robust and cost-effective manner will therefore play an integral part in the clinical translation of candidate gene therapies. In 2007 the UK Department of Health commissioned an analysis of international good manufacturing practice (GMP) gene therapy vector capacity and capability. Out of the eight organisations with LV production capability, the average cost of a LV batch (for 10 patients and including process development) was £242k - £469k (Watson, 2007).

LV production methods have changed little since the initial reports of lentiviruses as gene therapy vectors. For *in vitro* studies and *in vivo* experimentation in small animal models, relatively small vector quantities on the order of 10^9 transducing units (TU) are sufficient. The scale of production for clinical and commercial material will be dependent on the specific application. This is because the quantity of vector required is

influenced by factors such as the method of gene therapy administration (*ex vivo*/*in vivo*), disease pathophysiology, LV potency and the need for repeat doses (Andreadis et al., 1999). It has been estimated that early phase clinical trials for *ex vivo* therapy will require in the range of 10^{11}-10^{12} functional particles (Ansorge et al., 2010). In a report on the manufacture of LV for a WAS clinical trial for five patients, the aim was to produce 25×10^9 purified and formulated infectious particles (Merten et al., 2010). It was assumed that quality control would consume half of the batch and that the overall downstream recovery would be approximately 10%. Titres from transiently transfected HEK 293T cells are typically close to 10^7 TU mL^{-1}, which led to a requirement for cell culture to produce 5×10^{10} particles in 50 L of viral supernatant. For rare diseases the relatively large volume of harvested cell culture fluid required per patient is not likely to be an insurmountable problem. However, if T cell therapies deliver on their potential as a potent treatment for common cancers, vector COGs may become prohibitive to these therapies gaining reimbursement from public healthcare providers.

Despite the difficulties involved with generating high quality and high titre LV preparations, the possibility of generating a platform production system improves their suitability for commercialisation. Cell line development timelines can be reduced by either (a) the use of packaging cells, ready to be stably transfected with a transfer vector, or (b) use of systems, such as artificial chromosomes, that allow the introduction of the packaging and transfer plasmids in one transfection event. Upstream and downstream processing can likely be operated identically irrespective of the transfer vector, as the characteristics of the vector that matter for bioprocessing (except for bioreactor titre which is linked to the transgene (Al Yacoub et al., 2007)) will remain the same (provided the vectors are pseudotyped with the same Env). Once processes have been established that result in an economically favourable bioreactor titre and downstream recovery, the process development effort required could be on a par, or even smaller, than that required for monoclonal antibodies (mAbs) (especially when considering that e.g., IgG production can be significantly different to IgM production) produced from platform processes.

1.7 Upstream processing

The two routes for expression of LVs rely on either transient transfection of three to four plasmids, or production using stable packaging and producer cell lines (Warnock et

al., 2006). Packaging cell lines express all LV components except the transfer vector, which has to be transfected for vector production. Producer cell lines express all the components necessary for vector production, hence transfection is not required.

Variants of HEK 293 are the most widely used cell host for expression of LVs and other viral vectors (Stacey and Merten, 2011). Originally derived in 1977 by Graham et al., HEK 293 cells are now one of the most commonly used cell lines in expression experiments (Graham et al., 1977; Shaw et al., 2002). This cell type has been extensively used for therapeutic protein- and γ-retroviral vector-production and thus has a proven safe track record. The popularity of HEK 293 cells is primarily due to their high transfectability and ability to being adapted to suspension-growth in serum-free medium (Segura et al., 2007). In addition, they efficiently generate infection-capable virus particles (Smith and Shioda, 2009) and show moderate dependence on the HIV-1 accessory genes to generate infectious virus (Farson et al., 2001). Due to the human origin of HEK 293 cells, the glycosylation pattern of the envelope protein is of human type (Warnock et al., 2006). This is advantageous for *in vivo* therapy as vectors with non-human glycosylation are inactivated by the complement system within 20 min of administration.

It has been argued that HEK 293 should be described as a "cell family" rather than the "HEK cell" expression system, due to the large variety of HEK 293 descendants (Geisse, 2009). The most commonly used subvariant of HEK 293 for LV production is HEK 293T, which constitutively express the SV40 large T-antigen (Segura et al., 2007). This leads to increased expression levels by episomal maintenance and replication of the plasmid for plasmids carrying the SV40 origin of replication. In addition, the SV40 large T-antigen enhances nuclear import of expression vectors through nuclear targeting sequences (Geisse, 2009). The SV40 large T-antigen is an oncogene, thus the use of HEK 293T cells presents a safety concern (Merten et al., 2010). Merten et al. compared LV production using HEK 293T to HEK 293-based production. However, expression using HEK 293 cells could not be optimised to generate comparable levels of infectious LV to HEK 293T-based production, thus HEK 293T cells were used for generation of clinical material.

Other mammalian cell lines have also been used for LV expression, including HeLa, HT1080 and African green monkey kidney COS-1 cells (Ikeda et al., 2003; Smith and Shioda, 2009). The strong adherence of COS-1 cells to cell culture treated plastic compared to HEK 293T cells has been found to be beneficial as it leads to a

lower level of host cell contamination of the LV-containing supernatant (Smith and Shioda, 2009). This is advantageous for applications where downstream processing is impractical, however, for *in vivo* human therapy, some level of purification would be required to prevent immune and inflammatory responses, as well as to increase transduction efficiency (Segura et al., 2006).

Reported titres are typically on the order of 10^7 TU mL^{-1} (Ansorge et al., 2010), however, as discussed in Section 1.9, titre comparisons between research groups are hampered by the variations in titration method. LV titres are significantly lower than adenoviral vector titres, where suspension-adapted cells typically produce on the order of 10^{10} to 10^{11} viral particles mL^{-1} (Warnock et al., 2006).

1.7.1 Production of LVs using transient transfection

Due to the short development time, transient transfection of mammalian cells is commonly used for the production of biopharmaceuticals (Pham et al., 2006). The success of expression is determined primarily by the cell line, culture medium, expression vector, transfection reagent and the expressed protein (Geisse, 2009). HEK 293 cells have been the most commonly employed cell line, however, more recently Chinese hamster ovary (CHO) cells have also been utilised. The increased use of CHO cells is driven by the fact that this cell type is the predominantly used expression host for biopharmaceuticals, thus early research activities can be aligned with cell line development and product characterisation for production purposes.

The choice of transfection agent has implications for process economics, and for large-scale applications only calcium phosphate (CaPi) and polycation polyethylenimine (PEI) are economically viable options (Geisse, 2009; Pham et al., 2006). CaPi precipitation-mediated transfection, however, suffers from being highly sensitive to pH variations, thus process reproducibility is often poor (Kuroda et al., 2009). In addition, serum- or albumin-containing medium is required to reduce the cytotoxic effect of CaPi and complete medium exchange is required prior to transfection (Pham et al., 2006). For these reasons, CaPi is not suitable for large-scale production of clinical material.

For LV production, transient transfection offers significantly reduced development times and increased flexibility compared to the generation of stable packaging and producer cell lines (Ansorge et al., 2009). Furthermore, transfection-

based approaches allow use of cytotoxic and cytostatic transgenes and vector components. The majority of reported protocols rely on transient transfection of adherent cell lines (e.g., Kuroda et al., 2009) and have changed little since the first applications of lentiviruses to gene therapy in the late 1990s. A more desirable approach is transient transfection of suspension-adapted cell lines due to the ease of scale-up and the advantages associated with culture in controllable bioreactors (discussed in Section 1.7.4). This has been reported for production of AAV (Hildinger et al., 2007) as well as LVs (Ansorge et al., 2009; Segura et al., 2007). Segura et al. (2007) investigated optimisation of PEI-mediated transient transfection conditions in shake flasks operated in batch replacement mode. The parameters studied were plasmid ratios, harvesting times and cell density at the time of transfection. To demonstrate the scalability of the process, the best conditions identified in shake flask experiments were applied to culture in a 3 L stirred tank bioreactor (STR), also run in batch replacement mode. A comparison of viral titre before and after optimisation was not presented, however, the average titre, following optimisation, in shake flasks and STR was 2.3×10^6 and 1.1×10^6 TU mL^{-1}, respectively. In shake flasks a maximum cell density of 1.8×10^6 cells mL^{-1} was achieved on the fifth day of culture, whereas the maximum STR cell density of 7.0×10^6 cells mL^{-1} was achieved on the sixth, and final, day of culture. Batch replacement mode, however, is not a suitable mode of operation for large-scale applications. Continuous harvest of labile products, such as LVs, can be achieved by employment of bioreactors operated in perfusion mode (Shirgaonkar et al., 2004). This was reported by Ansorge et al. (2009), who studied the optimisation of a transient transfection process utilising PEI. The effect of cell density at the time of transfection, medium formulation and medium sodium butyrate concentration on LV titre was investigated. Following optimisation, maximum titres of 2×10^8 TU mL^{-1} were achieved in shake flasks operated in batch replacement mode, and the overall productivity was increased 150-fold compared to the initial conditions. The optimised culture conditions were applied to culture in a 3.5 L STR operated in perfusion mode; however, the maximum titre achieved of 8×10^7 TU mL^{-1} was lower than that in shake flasks. The overall increase in productivity compared to non-optimised conditions was also less pronounced than in shake flasks, with optimised conditions resulting in a 75-fold productivity increase. The discrepancy in culture performance between the two systems may be due to the difference in engineering environment experienced by the

cells. The authors did not use any scaling criteria, e.g., power input per unit volume, when transferring from shake flask- to STR-culture. In addition, batch-replacement mode in shake flasks is likely to be an unsatisfactory mimic of the perfusion device (an acoustic filter) used for STR-cultures.

A different approach was taken by Lesch et al. (2011) who used a recombinant baculovirus (BV)-based production system. This production method does not require high quantities of pDNA and may therefore be preferable to traditional transient transfection protocols. In addition, BVs have a practically unlimited transgene capacity. Suspension-growing HEK 293T cells were transduced with four BVs carrying the components necessary for LV production and cultured in 6-well plates and roller bottles. The highest titres achieved in 6-well plates were 1.2×10^5 TU mL^{-1}, whereas in roller bottles the highest titre reported was 2.2×10^6 TU mL^{-1}. These titres are significantly lower than pDNA-based approaches. Furthermore, the introduction of a second virus may be undesirable from a manufacturing perspective as downstream processing will need to demonstrate clearance of BV. From a process economics point of view, generating GMP-grade pDNA may be preferable to generating GMP-grade BV.

In addition to CaPi- and PEI-mediated transient transfection, a third economically viable alternative for production of LV by transient transfection is emerging in the form of flow electroporation. The technology, commercialised by MaxCyte, has been shown to be able to produce titres in the order of 10^8 TU mL^{-1} from a suspension growing cell line (Witting et al., 2012). Very encouragingly, this technique generated high infectivity values ($\approx 1.5 \times 10^5$ TU ng^{-1} of p24) which makes it very interesting for generation of clinical material.

1.7.2 Production of LVs using packaging and producer cell lines

Despite the advantages of LV production using transient transfection, there are a number of drawbacks that call for the development of stable packaging and producer cell lines. COGs are increased due to the need for large amounts of GMP-grade pDNA; process complexity is increased due to the additional transfection steps; batch-to-batch reproducibility is typically poor; and cotransfection may increase the risk of plasmid recombination (Ansorge et al., 2010; Farson et al., 2001). Batch variability is a regulatory concern, but it also affects downstream processing, as it is difficult to

develop a purification process that can produce product to the correct specification from harvest material of varying quality. Stable producer cell lines would greatly alleviate these issues. In addition, productivity is expected to be higher when using a stable cell line, as with transient transfection, cell expansion after transfection leads to dilution of the transiently transferred genetic material (Ansorge et al., 2010). Another benefit of stable producer cells is that harvested cell culture medium is, unlike material from transiently transfected cells, free from tubulovesicular structures containing pDNA that may stimulate DCs (Broussau et al., 2008).

A growing number of reports on stable LV packaging and producer cell lines have been published (Broussau et al., 2008; Cockrell et al., 2006; Farson et al., 2001; Ikeda et al., 2003; Kafri et al., 1999; Klages et al., 2000; Kuate et al., 2002; Ni et al., 2005; Pacchia et al., 2001; Sanber et al., 2015; Sparacio et al., 2001; Stewart et al., 2009; Stewart et al., 2011; Stornaiuolo et al., 2013; Throm et al., 2009; Wielgosz et al., 2015; Xu et al., 2001). The large majority of these publications describe packaging and producer cell lines for production of HIV-1-based vectors, with the exception of EIAV- (Stewart et al., 2009; Stewart et al., 2011) and SIV-producers (Kuate et al., 2002).

A key issue in the development of packaging and producer cell lines has been the cytotoxic and cytostatic effects of many of the LV components. The commonly used envelope protein VSV-G is highly cytopathic, presumably due to its ability to fuse intracellular membranes (Quinonez and Sutton, 2002). The HIV Rev protein has been shown to prevent or slow progression through mitosis (Miyazaki et al., 1995). The HIV protease is also known to be cytotoxic, most likely due to the fact that several cellular proteins (e.g., vimentin and Bcl2) have been demonstrated to be substrates for this enzyme (Kaplan and Swanstrom, 1991; Sparacio et al., 2001). Probably most critically, HIV protease has been shown to cleave procaspase 8, which initiates a process that results in apoptosis (Nie et al., 2007). It has been suggested (Segura et al., 2013) that cell lines should overexpress antiapoptotic genes to mitigate cytotoxic effects.

In the majority of stable cell lines, expression of cytotoxic components is placed under the control of the tetracycline response element (TRE) for inducible expression. Most commonly the tet-off system is used, where expression is induced upon the removal of doxycycline (dox) (a tet analogue). The two key disadvantages of this system are that high level expression is often only achieved several days after removal of dox and the need for medium exchange, thus making it unsuitable for use with suspension-growing cells (Ansorge et al., 2010). The tet-on system offers improved

practicability and has been successfully used (Broussau et al., 2008; Stewart et al., 2009). Ikeda et al. (2003) reported the development of a constitutively expressing producer cell line for a HIV-1-based vector. Cytotoxicity was reduced by pseudotyping the vector with γ-retrovirus Env instead of the commonly used VSV-G. However, the main difference in their method was the use of codon optimised *gag-pol* and *rev* constructs, which were delivered by γ-retroviral vectors. The reason for the success of this approach is not understood, but titres greater than 1×10^7 TU mL^{-1} during a prolonged period were reported. Recently, the same group reported the construction of another cell line, developed in a way that makes it suitable for GMP production of SIN LVs (Sanber et al., 2015). The titre obtained was approximately an order of magnitude lower than that reported for the previous cell line and as the vector is pseudotyped with an envelope of poor stability, a significant loss of titre may be observed during purification and concentration.

Concerns of transmissible spongiform encephalopathies (TSE) to humans through bovine products have led to efforts to remove bovine serum from biopharmaceutical production processes (e.g., EMEA/410/01 Rev. 2). So far, however, there has only been one publication describing a LV producer cell line growing in serum-free media (Broussau et al., 2008). This is also one of two reports of a suspension-adapted producer cell line – a desirable feature from a scalability perspective. In shake flask culture with daily medium exchange, the cell line resulted in a maximum titre of 3.4×10^7 TU mL^{-1} four days following induction, thus demonstrating the feasibility of scalable, animal component-free LV production.

Guy et al (2013) reported the optimisation of culture conditions for a suspension-adapted version of the producer cell line described by Stewart et al (2009, 2011). The authors utilised design of experiment (DoE) software for experimental design and analysis combined with high-throughput experimentation (microwell cultures). This illustrates that LV bioprocess research is maturing towards adapting the approaches used for already commercialised classes of biopharmaceuticals. The authors achieved a 2- to 3-fold improvement in titre compared to shake flask culture. The optimised titres were still lower than the titres obtained when the cell line was growing adherently, highlighting the difficulty of suspension adaptation of LV producer cell lines.

Optimisation of culture conditions has so far only resulted in modest improvements of LV titres. In a recent study, Rodrigues et al (2015) reported the 30-fold titre increase of an MLV vector produced by a stable producer cell line based on

HEK 293 cells following down-regulation of hypoxia inducible factor 1 and pyruvate dehydrogenase kinase. Metabolic engineering approaches such as these will hopefully result in LV titre improvements in the near future.

1.7.3 Medium development for improved LV production

Medium sodium butyrate supplementation is a well-established method to enhance expression of recombinant proteins in cell lines of mammalian origin (Geisse, 2009). Sodium butyrate acts by inhibition of histone deacetylase, resulting in histone hyperacetylation, which leads to chromatin remodelling and increased accessibility for transcription factors. Gene silencing of LV expression cassettes commonly occurs in both transient transfection systems and stable cell lines (Ansorge et al., 2010). Numerous reports exist where sodium butyrate in concentrations of 2-10 mM has been used to improve LV production. In cases where expression levels are already high, sodium butyrate has been found to not further improve protein production (Gasmi et al., 1999).

Chloroquine is an amine that raises the pH of endosomes and lysosomes, which may prevent degradation of transfected DNA (Karolewski et al., 2003). The effect of chloroquine is varied and in one study, utilising PEI-mediated transfection, it had a negative or negligible effect, depending on the culture medium used (Kuroda et al., 2009). Chloroquine is cytotoxic, in this case it is likely that cytotoxicity outweighed the beneficial effects. Titre reduction upon chloroquine addition was also reported for a CaPi-mediated transfection protocol (Karolewski et al., 2003). This was again attributed to chloroquine cytotoxicity and it was speculated that VSV-G sequestration within the endosomes led to the titre decline.

Ellis et al. (2011) reported titre increases of 3- to 8-fold using caffeine-supplementation following transient transfection. Addition of up to 4 mM caffeine 17 to 41 hours posttransfection was found to result in optimal titres. It was postulated that caffeine acts by inhibiting a group of kinases which are important signalling proteins involved in the repair of DNA double-stranded breaks.

Enveloped viruses such as influenza, retroviruses and Ebola bud from the membrane of the host cell and thus obtain a host cell-derived lipid bilayer (Ansorge et al., 2010). It has been suggested that sphingolipids and cholesterol separate in cell membrane microdomains termed lipid or membrane rafts. It has been hypothesised that

cholesterol stabilises these rafts from which virus budding takes place. In line with this theory, supplementation with cholesterol-containing mixtures has been reported to lead to an increase in LV infectivity (Chen et al., 2009; Mitta et al., 2005).

1.7.4 Bioreactor design and operation

To date, all LV material for clinical application has been generated by anchorage-dependent cells (Ansorge et al., 2010). Bioreactor designs compatible with adherent cell growth typically require an increase in the unit number of vessels (scale-out) rather than an increase in unit size (scale-up) for increased production. This leads to labour intensive processes that are unsuitable for large-scale production (Andreadis et al., 1999; Warnock et al., 2006). In addition, the most commonly used bioreactor types for production of clinical-grade vector, e.g., roller bottles and Nunc Cell Factories, only allow bioreactor operation in batch, fed-batch or batch replacement mode. This results in lengthy virus particle residence times, leading to vector inactivation. Another drawback of bioreactor designs for adherent cell culture is the difficulty of process monitoring and control. The control of key culture parameters, such as pH and dissolved oxygen (DO), is essential to achieve optimal cell growth and productivity. This is especially important for LV production, given the sensitivity of enveloped viruses to environmental conditions.

However, there is the possibility of culturing anchorage-dependent cell lines on microcarriers, thus enabling growth in conventional STRs. Microcarriers are available in either porous or solid formats, both of which have been used for γ-retroviral vector production (Warnock et al., 2006). The use of porous forms has the advantage of larger surface area, thereby enabling growth to higher cell densities. Mass transfer limitations, however, lead to heterogeneous environments and increased virus particle residence times. For γ-retroviral vector production, solid microcarriers have been found to result in titres comparable to traditional static cultures, thus the benefits associated controllable culture did not lead to increased productivity (Wu et al., 2002). Throm et al. (2009) reported culture of adherent HEK 293T producer cells growing on fibrous discs (Fibra-Cel® Disks, New Brunswick, Cambridge, UK) in a 5 L wave-type bioreactor. Following induction of the producer cells by removal of dox, LV was harvested daily by medium replacement, i.e., the bioreactor was operated in batch

replacement mode. Viral titre peaked at 1.9×10^7 TU mL^{-1} on the fourth day following induction.

The problems associated with intraparticle convection in porous microcarriers can be alleviated by using packed or fluidised bed bioreactors. These types of systems, along with fixed bed bioreactors, have been described as the most promising systems for γ-retroviral vector production (Warnock et al., 2006). Hollow fibre bioreactors have also been investigated for γ-retroviral vector production, but are associated with a number of disadvantages such as diffusion gradients. Sheu et al., 2015 recently reported the use of a hollow fibre bioreactor (Terumo BCT's Quantum Cell Expansion System) for LV production. The system used generated a vector titre comparable to that from tissue culture flasks and cell factories.

A limitation of packed bed, fixed bed and hollow fibre bioreactors is their limited scalability (Warnock et al., 2006). For example, the largest CellCube fixed bed bioreactor module has a culture area of 34 m^2. The iCELLis fixed bed bioreactor has been used for γ-retroviral vector production (Wang et al., 2015), and may be an interesting option for LV manufacturing as it is a single-use system with a maximum culture area of 500 m^2.

There have only been a few reports of LV production from suspension-growing cells (Ansorge et al., 2009; Broussau et al., 2008; Guy et al., 2013; Lesch et al., 2011; Segura et al., 2007; Segura et al., 2010; Witting et al., 2012, Thomas et al., 2013). As discussed in Sections 1.7.1 and 1.7.2, maximum viral titres in the most promising of these studies were in the order of 10^7 TU mL^{-1} or above, thus demonstrating that scalable LV production in STRs is achievable. Due to the temperature sensitivity of LVs, perfusion culture has been described as a promising option for LV manufacturing. Perfusion would allow continuously harvested vector to be refrigerated or further processed instead of risking potential vector inactivation at the elevated bioreactor temperature. However, only one published report describes the application of perfusion culture to LV production (Ansorge et al., 2009). This study employed an unoptimised perfusion rate of one reactor volume per day. For γ-retroviral vector production, a perfusion rate of three to four reactor volumes per day has been suggested as optimal, thus studies looking at the optimal rate for LV production may lead to further titre increases.

Guy et al. (2013) used a wave-type bioreactor for LV production. This type of bioreactor appears likely to increase in popularity for LV cultures as Lesch et al. (2011)

report using a suspension-adapted cell line in roller bottles for small-scale studies with a view to use a wave-type system for large-scale production. Roller bottles were chosen as the small-scale system as the mixing characteristics were expected to be similar to those produced by wave-induced mixing. The increased use of wave-type systems may be due to the fact that it is a single-use bioreactor (Brecht, 2009). Single-use processing equipment does not require cleaning-in-place and steam-in-place; this leads to advantages such as increased flexibility due to short changeover procedures, reduced validation effort, and cost savings due to reduced need for steam, water and cleaning solutions. The initial capital investment is also reduced and operational flexibility, e.g., process design and scale, is increased. In addition, the gentle hydrodynamic environment provided by wave-induced motion and bubble-free aeration may be beneficial for production of labile products such as LVs.

1.8 Downstream processing

Downstream processing of LVs is required for two important reasons: (i) virus concentration following cell culture is too low for many gene therapy applications and (ii) contaminant and impurity removal is necessary to increase product safety and potency (Segura et al., 2006). Gene therapy is still in its infancy and regulatory authorities have no strict guidelines regarding LV manufacturing (e.g., EMA, 2005). It seems likely that as more clinical data becomes available and products get closer to commercialisation, these guidelines will become firmer. Purity specifications are likely to depend on whether the therapies are *ex vivo* or *in vivo*, with *ex vivo* treatments possibly having reduced purity requirements. A number of thorough reviews of γ-retroviral and LV downstream processing have been published (Rodrigues et al., 2007; Segura et al., 2006; Segura et al., 2010; Segura et al., 2013).

Table 1.1 provides an overview of the impurities typically present in a LV preparation. The main impurities following cell culture are typically added materials, including serum proteins and pDNA (if transient transfection is used), and host cell-derived components, e.g., whole cells, cell debris, host cell proteins (HCPs) and nucleic acids. High molecular weight proteoglycans and DNA are particularly challenging to remove as they, like LVs, are large and carry a strong net negative charge.

So far, LV titres following cell culture have been low, leading to a requirement for downstream processing to handle relatively large volumes. Possible increased use of

perfusion culture will lead to even larger total processing volumes. In addition to this, total downstream recoveries are typically low with reports of overall yields between 1% and 30% (Merten et al., 2010; Slepushkin et al., 2003). Downstream processing operations therefore need the capacity to handle large volumes and the development of scalable purification strategies becomes imperative. It is also worth pointing out that process robustness is an issue, with authors reporting variable overall recoveries for the same process (Merten et al., 2011). At this early stage of development and for therapies for life-threatening conditions, such as MLD, vector process robustness may not be a red flag for regulators, however, as LV technology matures, process understanding via quality by design (QbD) approaches, may become an expectation.

Product-related impurities	**Process-related impurities**
Inactive vector forms Viral aggregates Env(-) particles Free viral components Soluble Env protein Broken/disassembled particles	Culture medium derived • Proteins, peptides, amino acids • Lipids, phospholipids • Salts, buffers • Sugars • Trace elements, vitamins • Serum/hydrolysate additives Host cell derived • Host cell proteins • Host cell nucleic acids • Cell vesicles, debris • Proteoglycans Production derived • pDNA • Transfection reagent, e.g, PEI Purification derived • Nucleases • Buffers • Detergents • Leachables and extractables

Table 1.1 Product- and process-related impurities commonly found in LV production processes. Adapted from Segura et al., 2013.

Figure 1.4 illustrates an attempt at a generic downstream processing sequence for LV production. However, process sequence varies between institutions, as demonstrated in Table 1.2. As a rule of thumb for process developers, a downstream process with as few steps and buffer composition changes as possible is important in order to retain vector infectivity (Segura et al., 2011).

In Table 1.2 it can be seen that downstream processes for production of clinical-grade LV have included an endonuclease treatment step for pDNA digestion. This adds to the downstream processing time and cost; also, since the added enzyme must be removed, the complexity of the remaining steps is increased. Given the fragility of LVs, it would be desirable to avoid this extra step. This may be another reason for using a producer cell line instead of transient transfection approaches.

Most commercially available purification techniques employed in the biotechnology industry were originally developed for protein separations (Segura et al., 2005). Compared to proteins, viruses are large and poorly defined, which leads to downstream processing becoming an even greater challenge than it is for proteins.

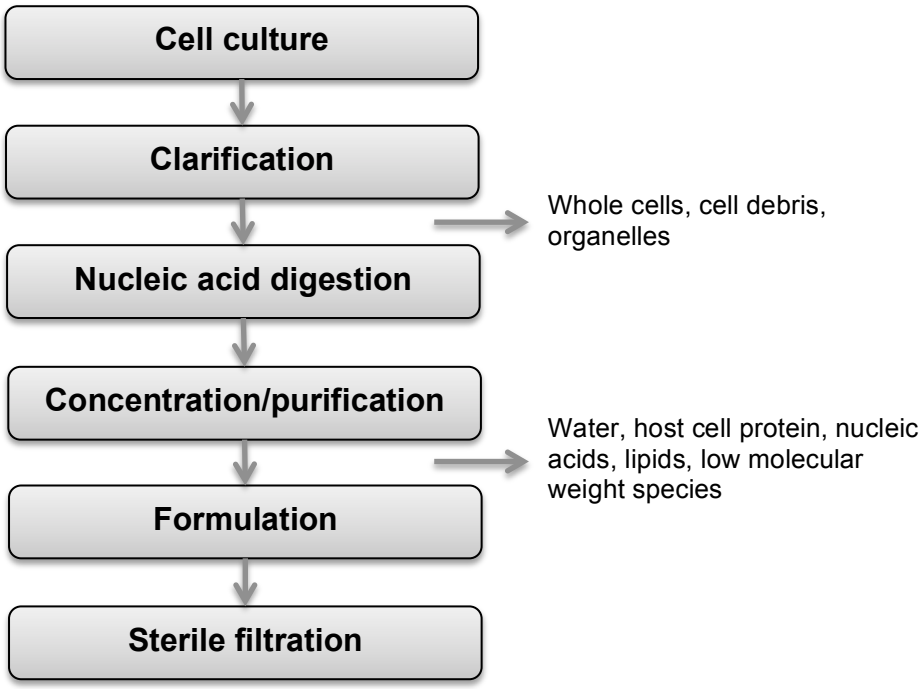


Figure 1.4 Generic flowsheet for LV downstream processing.

Institution	Process steps in chronological order						Ref.
VIRxSYS (MD, USA)	Membrane filtration	AEX membrane chromatography	UF/DF	Nucleic acid digestion	DF	Sterile filtration	Slepushkin et al., 2003
Oxford Biomedica (Oxford, UK)	Membrane filtration	Nucleic acid digestion	AEX membrane chromatography	UF/DF	Sterile filtration	UF/DF	Truran et al., 2009
Généthon (Évry, France)	Membrane filtration	Nucleic acid digestion	AEX chromatography	UF/DF	SEC	Sterile filtration	Merten et al., 2011
Beckman Research Institute (CA, USA)	Membrane filtration	Nucleic acid digestion	UF/DF	High-speed batch centrifugation	Batch centrifugation		Ausubel et al., 2012
MolMed (Milan, Italy)	Depth and membrane filtration	Nucleic acid digestion	AEX chromatography	UF	SEC	Sterile filtration	Aiuti et al., 2013
Indiana University (IN, USA)	"Clarification"	Concentration	Nucleic acid digestion	DF			Cavazzana-Calvo et al., 2010

Table 1.2 Summary of LV downstream processes for manufacturing of clinical-grade material. Adapted from Merten et al., 2014.

1.8.1 Clarification

At the laboratory scale, removal of whole host cells and debris is done by low speed centrifugation and microfiltration (Segura et al., 2006). The centrifugation step helps reduce fouling of the 0.45 μm pore size membrane which is typically used for final clarification. At scales greater than 1 L, a single microfiltration step is preferred, but may lead to rapid clogging of the membrane, resulting in virus retention and thus reduced recovery. Studies investigating the optimum membrane area per unit volume of feedstock are therefore valuable. For preparation of clinical-grade material, a depth pre-filter (retention rating > 1 μm) followed by a series of membranes of decreasing pore size (typically 0.8 μm followed by 0.45 μm) is the preferred option (e.g., Biffi et al., 2013; Merten et al., 2010; Slepushkin et al., 2003). For protein purification, a 0.22 μm membrane filtration step is typically the final part of clarification, however, for LVs, 0.22 μm filtration tends to result in significant product loss. Due to these large product losses with sterilising-grade filters, there are now published examples of organisations focusing on closed processes and omitting sterile filtration from GMP manufacturing processes (Ausubel et al., 2012), or concentrating the product after sterile filtration (Truran, 2009). However, from a regulatory point of view, this may be a risky strategy as product sterility assurance becomes more difficult.

Tangential flow microfiltration has not been reported for LV clarification, but may present an alternative to the cascade of filters of decreasing pore size commonly used. The vast majority of scalable, commercially available depth filters are positively charged (Roush and Lu, 2008), thereby providing DNA removal. The positive charge, however, means that the filters filters bind the negatively charged LVs.

1.8.2 Concentration and buffer exchange

Concentration of the feedstream at an early stage during downstream processing is beneficial as it reduces the feed volume for subsequent steps (Segura et al., 2006). Centrifugal methods have been widely used for LV concentration. Ultracentrifugation at 20,000-90,000 *g* as well as low-speed methods can be used to efficiently separate LV particles. Concentration factors greater than 100 can be achieved, but transduction efficiencies have not been found to increase proportionally with the concentration factor. This loss in functional virus has been ascribed to hydrodynamic shear forces,

long processing times and co-concentration of virus with inhibitors of transduction. The vector pseudotype will largely determine the suitability of concentration by centrifugation, which is a reason for the popularity of the VSV-G Env (Burns et al., 1993). Drawbacks of ultracentrifugation include process variability as a result of difficulties related to reproducible resuspension of the virus pellet and the limited volume capacity of ultra-high speed rotors (Zhang et al., 2001). In addition, cell debris and high molecular weight impurities that co-concentrate with virus will need to be removed in subsequent processing steps (Transfiguracion et al., 2003).

Precipitation has been used for concentration of lentiviral particles (Pham et al., 2001; Zhang et al., 2001). Following precipitation, virus can be rapidly recovered by low-speed centrifugation. Zhang et al. (2001) employed the cationic polymer poly-L-lysine as a precipitating agent. Two important limitations of this technique are that (i) the polymer was seen to interact irreversibly with the virus and (ii) the low product yield of 26%. Pham et al. (2001) used CaPi-mediated precipitation followed by centrifugation and EDTA treatment to re-dissolve virus pellets. EDTA was found to inactivate virus particles, but this problem was alleviated by immediate dialysis following EDTA treatment and product recoveries of 50-60% were reported.

Ultrafiltration (UF) allows for the use of gentle processing conditions, e.g., in contrast to centrifugation and precipitation it does not require a change of phase (Segura et al., 2006). It is also a highly scalable technology with a potentially high purification factor. In addition, it can be combined with diafiltration (DF) for buffer exchange or formulation. From Table 1.2 it is clear that UF is the method of choice for concentration of LV material intended for clinical studies. For γ-retroviral and LV processing, molecular weight cut-offs of 100 to 500 kDa are typically used (Ausubel et al., 2012; Bandeira et al., 2012; Cooper et al., 2011; Merten et al., 2011). Membrane materials that have been used include regenerated cellulose, polysulfone and polyethersulfone (PES). The earliest report of the employment of UF for lentivirus processing involved the concentration of wild-type HIV-1 (Makino et al., 1994). This study used a hollow fibre membrane configuration, which has subsequently been used for the generation of clinical-grade LV (e.g., Merten et al., 2010; Slepushkin et al., 2003).

1.8.3 Purification

Lentiviruses share similar structural, physical and biochemical properties with γ-retroviruses; therefore, reports on γ-retrovirus purification may be valuable for the development of LV processes. Density gradient ultracentrifugation has been established as a powerful method for the purification of γ-retroviruses (Segura et al., 2006). Unlike other purification techniques, it offers the potential to separate virus particles from closely related species, such as defective vectors and cell membrane vesicles. However, this method is difficult to scale up, is labour-intensive and has long processing times (Segura et al., 2005).

1.8.3.1 Chromatography

Chromatography has become the workhorse of most industrial-scale protein separation processes due to its scalability, consistency, fast processing times and amenability to automation. LV purification has traditionally been carried out by density gradient ultracentrifugation, but chromatography-based methods have been explored as a more feasible large-scale purification option. Platform purification processes that suit different viral vectors are not yet available like they are for mAb production (e.g., Farid, 2006). An initial screening of chromatographic media is therefore always needed (Rodrigues et al., 2007). The selection of suitable resins is complicated by the fact that little is known about the composition of the retroviral membrane (Segura et al., 2006).

1.8.3.1.1 Chromatography stationary phases

Typically, the stationary phase used for chromatography is particle-based, but due to the large size of viruses, there has been growing interest in the use of monolithic resins and membrane adsorbers for virus bioprocessing (Jungbauer and Hahn, 2008). Monolithic adsorbents are highly porous, allowing mass transfer to occur predominantly by convection, which results in retention of dynamic binding capacity and resolution at high flow rates (Barut et al., 2005; Jungbauer and Hahn, 2008; Podgornik et al., 2000). The compatibility of monoliths with high flow rates means that processing times can be kept short, which should enable better retention of LV infectivity. For particle-based adsorbents, the majority of binding sites are located in small, internal pores, into which mass transfer is limited by diffusion. These pores are typically smaller or in the same

size order as the diameter of LVs, thus binding will occur only on the surface of the beads, resulting in poor ligand utilisation. There is evidence that for viruses the size of LVs, even perfusive bead-based resins, where the pore size is several times that of the virus hydrodynamic diameter, particles do not enter the pores (Trilisky, 2009). This is hypothesised to be due to particles first binding on the surface of the beads and thereby blocking entrance to the pores.

1.8.3.1.2 Anion exchange chromatography

Most viruses carry a net negative charge at neutral pH (Michen and Graule, 2010), which makes purification of LVs by anion exchange (AEX) chromatography a suitable option.

Table 1.3 presents a summary of published reports of AEX chromatography for VSV-G (and measles virus glycoproteins for Marino et al., 2015) pseudotyped LVs. Reported yields span a wide range, but it should be pointed out that chromatography yields depend on how fractions were collected and there is a trade-off between yield and purity. Both strong (quaternary ammonium (Q)) and weak (diethylaminoethyl (DEAE)) anion exchange ligands have been used with similar recoveries and purification factors. The 80% product recovery reported by Bandeira et al. (2012) is the highest bind-and-elute yield reported for a LV. The use of a five-fold dilution immediately following elution led to retention of infectious titre (24% higher than if no dilution was used). This illustrates the need to keep the inherent fragility of LVs in mind when developing purification processes.

In the instances where membrane-based and monolithic resins have been compared, the recoveries have been in favour of monolithic resins (Bandeira et al., 2012; Lesch et al., 2011). Authors have not speculated as to why this is, however, membranes have been developed and optimised for polishing of protein products in flow-through mode. This is only true of comparative studies as several authors report good LV yields from membrane adsorbers. For example, Marino et al. (2015) used Pall Acrodisc membranes to capture LVs pseudotyped with measles virus glycoproteins and report recoveries up to 80% following step elution from a membrane operated by an ÄKTA chromatography system.

With the general aims of as few processing steps as possible and a minimum number of buffer changes, clarified LV is typically loaded directly onto AEX resins.

Viral particles contain multiple binding sites and therefore tend to have enhanced binding strength to AEX resins compared to contaminating proteins such as HCPs and free vector components (Segura et al., 2011). LV loading onto AEX supports can therefore be carried out at high ionic strength, resulting in increased selectivity and dynamic binding capacity (DBC).

Ligand chemistry	Type of stationary phase	Yield (%)	Elution type	Reference
DEAE	Membrane (Fractoflow DEA 80-6, Merck KGaA)	45	Gradient	Scherr et al., 2002
DEAE	Membrane (Sartobind D MA75, Sartorius, Epsom, Surrey)	29	Gradient	Lesch et al., 2011
DEAE	Monolith (CIM DEAE, Bia Separations, Villach, Austria)	65	Gradient	Lesch et al., 2011
DEAE	Monolith (CIM DEAE, Bia Separations, Villach, Austria)	80	Step	Bandeira et al., 2012
DEAE	Membrane (Sartobind D MA75, Sartorius, Epsom, Surrey)	28	Step	Bandeira et al., 2012
DEAE	Bead (Specific resin not stated, Tosoh Bioscience, Tokyo, Japan)	Not available	Step	Merten et al., 2011
Q	Bead (HiTrap Q, HP, Amersham Biosciences)	50	Gradient	Yamada et al., 2003
Q	Membrane (Mustang Q, Pall, Portsmouth, UK)	56	Gradient	Kutner et al., 2009
Q	Membrane (Sartobind Q, Sartorius, Epsom, Surrey)	56	Step	Zimmermann et al., 2011
Q	Membrane (Mustang Q, Pall, Portsmouth, UK)	60	Gradient	Marino et al., 2003
Q	Membrane (Mustang Q, Pall, Portsmouth, UK)	65	Step	Slepushkin et al., 2003
Q	Membrane (Mustang Q, Pall, Portsmouth, UK)	80/65	Step/gradient	(Marino et al., 2015)

Table 1.3 Summary of AEX chromatography of LVs.

1.8.3.1.3 Affinity chromatography

The limited knowledge of the LV membrane composition limits the possibility of using affinity adsorbents, which is otherwise an attractive option as it is a highly selective technique. However, it is possible to engineer LVs to express affinity tags on the virus surface. Cheeks et al. (2009) purified a histidine-tagged LV by immobilised metal affinity chromatography (IMAC). The study used a monolith as the stationary phase material and a yield of 69% was reported when a column from BIA Separations was used. Affinity tagged vectors are likely to be of limited use for products with intended clinical use due to regulatory hurdles associated with the use of vectors that have been engineered in this manner (Rodrigues et al., 2007).

Segura et al. (2007) evaluated heparin affinity chromatography for LV purification. Heparin is structurally related to the cell surface receptor heparan sulphate, which is used for cell binding by several viruses. In this study a yield of 53% was obtained and 94% of protein impurities were removed, along with 56% of DNA.

1.8.3.1.4 Size exclusion chromatography

Size exclusion chromatography (SEC), or gel filtration, is a non-adsorptive technique that has limited resolution, an inherent low capacity and works with low linear flow rates (Segura et al., 2006; Rodrigues et al., 2007). It may therefore mostly be useful as a polishing step, when the process volume has been sufficiently reduced. It is used in two of the clinical processes outlined in Table 1.2, however, in both instances, it is used for buffer exchange into formulation buffer, rather than purification, and it seems likely that diafiltration will replace SEC in many commercial processes.

1.8.4 Formulation

Possibly due to the many challenges of LV bioprocessing, very little has been published on LV formulation to date. The concentration challenge for LVs is not as significant as for e.g., mAbs, for which a large dose needs to be injected into patients (Daugherty and Mrsny, 2006). LVs are typically used in *ex vivo* treatment protocols and if a high multiplicity of infection (MOI) is required for transduction, a large volume of vector can simply be added rather than adding a highly concentrated vector. LVs for clinical trials have been formulated at a concentration one or two orders of magnitude higher

than the cell culture titre, i.e., at a concentration of 10^8-10^9 TU mL^{-1} (Aiuti et al., 2013; Biffi et al., 2013).

LVs for clinical protocols have been formulated as liquids, stored at ≤ -65°C and transported in the frozen state (Fan et al., 2013). As for concentration, *ex vivo* treatments lead to increased flexibility with respect to the formulation buffer. The transduced cells are washed before reinfusion into the patient, hence it is possible and convenient to formulate the vector into the medium used for transduction. Commonly used media are Lonza's X-VIVO™ and CellGenix's CellGro® (Aiuti et al., 2013; Biffi et al., 2013; O. W. Merten et al., 2011). Both contain proteins and sugars and are therefore likely to offer some cryoprotection. In a report from a contract manufacturing organisation (CMO), LV was formulated in phosphate buffered saline (PBS) with 0.04 g L^{-1} of lactose (Ausubel et al., 2012). A generic formulation buffer is likely to be practical for a CMO whose customers may use different transduction media. Fan et al. (2013) have submitted a patent application for histidine hydrochloride-based LV formulation buffers. In the application, example formulations maintained up to 74% of the original vector titre after 8 days at 25°C. Carmo et al. (2008) found that MLV vectors could be stabilised in liquid formulations containing recombinant human serum albumin (HSA), whereas for a LV, HSA along with a lipoprotein solution derived from bovine serum was required to improve stability compared to a Tris-based buffer. This result is interesting as it highlights that LVs are more difficult to stabilise than γ-retroviral vectors, however, animal-derived components are undesirable from a Chemistry, Manufacturing and Controls (CMC) perspective and other methods of LV stabilisation would have to be used for commercialised treatments.

1.9 Lentiviral vector quantification

Reliable methods for quantification of LVs are needed to support process development and to enable quality control of clinical trial material and, possibly soon, commercial manufacturing. The four broad categories of available techniques for virus quantification are (i) determining the titre of infectious particles, (ii) measuring the presence or function of viral proteins, (iii) detecting the presence of viral or marker nucleic acid within the viral genome and (iv) counting physical viral particles, whether labelled or unlabelled (Heider and Metzner, 2014).

The amount of infectious particles in a LV preparation, reported as the number of transduction-competent lentivirus particles per mL of virus stock, is determined by limiting dilution of the vector followed by transduction of target cells and finally flow cytometric measurement of marker protein expression (Geraerts et al., 2006). The most commonly used reporter protein is enhanced green fluorescent protein (eGFP), which allows straightforward analysis of functional titre by flow cytometry. This method, however, does not distinguish between single and multiple vector integrations. The cells chosen for transduction can be either of the same type as the therapeutic target cells or a universally used cell line, e.g., HEK 293, which facilitates titre comparisons (Ansorge et al., 2010). Titre comparison between research groups is complicated as transduction efficiency varies depending on the cell type chosen for titration (Zhang et al., 2004). In addition, other transduction conditions, such as inoculum volume and cell density, also affect titre determination and titre differences as high as 50-fold have been observed.

For LV, the viral protein most commonly assayed for is the p24 capsid protein (only applicable to HIV-based vectors), which can be quantified by an enzyme-linked immunosorbent assay (ELISA). Due to the use of antibody technology, high specificity and sensitivity are achieved with this assay, however, it is less stringent than measurement of transduction capability as the p24 protein is not always necessarily linked to a LV particle (Heider and Metzner, 2014). The p24 ELISA is significantly faster than cell-based methods as these take several days, but still require at least half a working day to complete. p24 determination gives an indication of the total number of LV particles and the ratio of p24 mass and transducing units (ng of p24:TUs), termed infectivity (or P:I ratio), is useful for indicating the quality of a LV preparation.

Quantitative polymerase chain reaction (qPCR) methods can be used in different ways to determine LV titre (Geraerts et al., 2006). Determining the number of integrated proviral DNA copies per cell allows the calculation of the number of functional particles. However, not all of these proviral DNA copies will lead to transgene expression and a more accurate count of functional particles can be obtained by determining the amount of lentiviral mRNA. qPCR techniques tend to overestimate the functional vector titre, which has led to the use of these methods being mostly limited to normalisation of vector preparations prior to transduction; screening of high producer clones during development of producer and packaging cell lines; vector optimisation; and to evaluate the quality of vector preparations (Ansorge et al., 2010).

Recently, a number of techniques that directly count physical viral particles have been developed and commercialised: field-flow fractionation multiple-angle laser light scattering (FFF-MALLS), nanoparticle tracking analysis (NTA), "flow virometry" using a VirusCounter device and tunable resistive pulse sensing (TRPS) (Heider and Metzner, 2014).

1.10 Thesis aim and objectives

The cost of vector production has been identified as one of the main challenges for successful clinical translation of gene therapies (Waehler et al., 2007). For commercial processes, highly pure vector preparations will be necessary, leading to a requirement for efficient downstream processing strategies. Patient dosages are likely to be above 10^{10} TUs, which, when coupled with low cell culture titres of 10^7 TU mL^{-1}, results in a need for downstream processes capable of handling large volumes of cell culture fluid (Rodrigues et al., 2007). Ultracentrifugation-based methods have been extensively used for concentration and purification of γ-retroviral vectors and LVs. However, these techniques are time-consuming, have limited scalability and lead to vector inactivation. Membrane- and chromatography-based purification strategies have been described as the most promising for γ-retroviral vector production (Rodrigues et al., 2007; Segura et al., 2006). To date there have been a limited number of reports of application of these types of unit operations for LV bioprocessing. In addition, the published studies typically involve a low level of bioprocess optimisation. This may be a consequence of researchers not using sufficiently scaled down models, thus making it time-consuming and resource-intensive to look at the effect of a large number of process parameters. High-throughput experimentation at the microscale could aid in selection of operating conditions, while limiting the time and resources spent on design space exploration (Chhatre and Titchener-Hooker, 2009; Titchener-Hooker et al., 2008). In addition, there is only one published report (Guy et al., 2013) of a study employing statistical DoE for LV process development. If any optimisation is reported, the one-factor-at-a-time (OFAT) method is used, which is resource-intensive and assumes that all variables are mutually independent (Islam et al., 2007). The combination of high-throughput experimentation at the microscale with DoE would be beneficial for LV process development. The benefits are not only associated with higher product yields, but this

approach will also result in enhanced process understanding, which, via the QbD initiative, is becoming a regulatory requirement (Scott, 2011).

The importance of scalable cell culture operations for LV production has led to an interest in suspension-adapted cell lines grown in STRs (Ansorge et al., 2010). Initially it was expected that the adherently growing cell lines used in this work would be possible to adapt to suspension growth. A serum concentration of 10% is typically used for LV production, which leads to serum components being the main source of impurities in harvested cell culture fluid (Segura et al., 2007). This complicates downstream processing, and it is possible that upstream titre improvements due to the use of serum are overshadowed by decreased product recovery during downstream operations. For regulatory reasons it is desirable to avoid the use of animal-derived components due to the risk of introducing adventitious agents and to reduce lot-to-lot variability (Whitford, 2005). Serum components also aid in cell adhesion and it is therefore advantageous to reduce the amount of serum added to the medium prior to attempting suspension adaptation. It was therefore an objective to reduce the medium serum concentration, followed by adaptation of the cells to suspension culture.

The lack of scalable and optimised downstream processing strategies for LV production forms the motivation for this project. Ultra-scale down (USD) methodologies in combination with automation will be used where possible to increase experimental throughput while reducing the time and resources expended. The aim of the project is to develop a scalable downstream processing route for a non-VSV-G-pseudotyped HIV-1-based model LV expressed by a producer cell line (Ikeda et al., 2003). The objectives of the project are:

- to adapt the packaging and producer cell lines to suspension growth (Chapter 2);
- to develop a harvest and concentration methodology capable of producing sufficient material for purification and formulation studies (Chapter 2);
- to investigate chromatography-based purification options in a high-throughput manner (Chapter 3); and
- to assess vector stability and formulation alternatives (Chapter 4).

2. VECTOR PRODUCTION, HARVEST AND CONCENTRATION

2.1 Introduction

To date, manufacturing of LVs for clinical application has been carried out with adherently grown HEK 293T cells, typically cultured in Nunc Cell Factory 10-tray stacks (e.g., Aiuti et al., 2013; Biffi et al., 2013; Merten et al., 2011). This scale-out approach has been practical for the small batch sizes produced, often resulting in enough vector material to treat less than five patients. For larger clinical studies and commercial supply, a more economical upstream process, based on volumetric scale-up, would be beneficial (Merten et al., 2014). Therefore, in this chapter, an attempt at adapting LV packaging and producer cell lines to suspension growth was made. Adherent cell growth was characterised to improve process understanding and to provide a baseline to compare any process modifications to.

To prepare material for chromatography (Chapter 3) and formulation (Chapter 4) studies, cell culture fluid (CCF) clarification and concentration was required. Harvesting of clinical LV material is typically carried out with a depth pre-filter, followed by a series of membranes of decreasing pore size (typically 0.8 μm followed by 0.45 μm) (e.g., Biffi et al., 2013; Merten et al., 2010; Slepushkin et al., 2003). Small-scale research experiments tend to omit the pre-depth filtration step and utilise a filtration unit with a built-in pre-filtration 0.8 μm membrane, followed by a 0.45 μm membrane (e.g., Bandeira et al., 2012). In general, researchers tend to not measure vector titres of unclarified stocks and therefore there are very few reports of clarification yield in the literature. In this work, different membrane materials and pore sizes were evaluated to ensure maximum recovery of infectious LV particles. For experiments requiring low volumes of material, syringe-driven filtration had sufficient throughput. In cases where larger volumes were needed, clarification using a Stericup device quickly generated the required volumes in a contained and aseptic manner.

Concentration of LVs using centrifugal filters has been reported both for research-grade (Bandeira et al., 2012; Zimmermann et al., 2011) and pre-clinical/clinical-grade material (Benati, 2012). Two molecular weight cut-off (MWCO) membranes were compared based on the recovery of infectious LV particles following 10-fold concentration.

Therefore, this chapter's aims and objectives are:

- to describe an attempt to adapt LV packaging and producer cell lines to suspension growth;
- to determine a suitable clarification filter for primary recovery; and
- to develop a concentration step capable of producing material for chromatography and formulation studies.

2.2 Materials and methods

2.2.1 Cell culture

Four cell lines were used in this work: HEK 293FT, 57R10E, EP1 and STAR-RDpro. All cell lines were kindly provided by the Division of Infection and Immunity, University College London.

2.2.1.1 HEK 293FT

HEK 293FT (Invitrogen, Cergy-Pontoise, France) is based on the HEK 293 cell line (Graham et al., 1977), but also expresses the SV40 large T antigen that allows for episomal replication of transfected plasmids containing the SV40 origin of replication.

2.2.1.2 57R10E

57R10E is a LV packaging cell line based on HEK 293FT (described by Knight, 2012). 57R10E was created by stably transfecting HEK 293FT with, in order, *gag-pol* (transduced), *rev* and *env* (RDpro). RDpro is the envelope protein of the retrovirus RD114, but with the addition of an HIV protease site at the R-peptide cleavage site (Ikeda et al., 2003).

2.2.1.3 EP1

EP1 is an LV producer cell line based on 57R10E that has been stably transfected with a transfer vector, which in this case is the SIN pHV vector shown in Figure 2.1.

2.2.1.4 STAR-RDpro

STAR-RDpro is a constitutive LV producer cell line that also has the RDpro envelope protein (described in Ikeda et al., 2003).

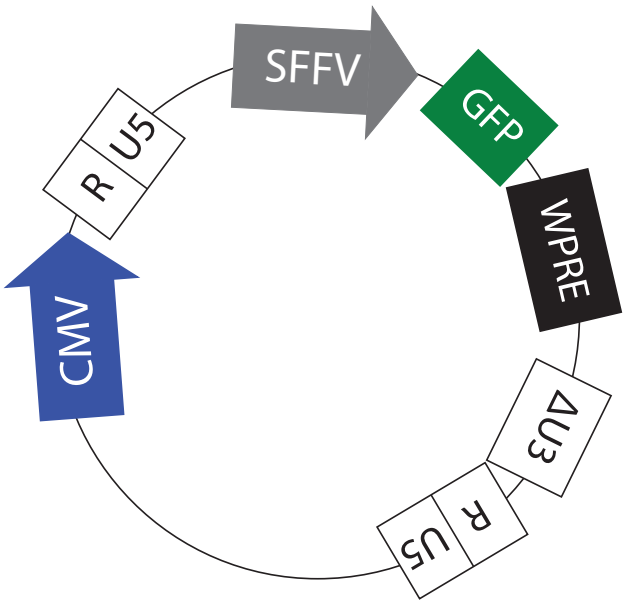


Figure 2.1 Schematic of SIN pHV vector used to generate the stable producer cell line, EP1. Abbreviations: CMV cytomegalovirus promoter, SFFV spleen focus-forming virus promoter.

2.2.1.5 Propagation of adherent cell lines

The adherent cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, USA) with 10% (v/v) fetal bovine serum (FBS; SAFC Biosciences, Andover, UK), 0.5% (v/v) penicillin-streptomycin (Life Technologies, Carlsbad, USA) and 2 mM L-glutamine (Life Technologies, Carlsbad, USA) (this will be referred to as DMEM-based medium). This medium contains phenol red as a pH indicator. Phenol red binds irreversibly to anion exchange chromatography resins at pH > 7 (e.g., GE Healthcare) and for this reason a phenol red-free medium was evaluated. The most similar off-the-shelf medium (Life Technologies, Carlsbad, USA) was selected and supplemented with additional L-glutamine and sodium pyruvate (Life Technologies, Carlsbad, USA) to give the same concentrations as in the DMEM-based medium. FBS and penicillin-streptomycin was added in the same way as for DMEM-based medium. DMEM with phenol red was used in Sections 2.3.1, 2.3.2 and 2.3.3. Both media were used in Section 2.3.4. Finally, DMEM without phenol red was used for the work reported in Sections 2.3.5 and 2.3.6.

Cells were cultured in tissue culture flasks (Nunc, Roskilde, Denmark) in a 37°C and 10% CO_2 incubator (Sanyo, Loughborough, UK). Subculturing was routinely carried out at 2-3 day intervals – when cells were 80-90% confluent – using a 1:4 or 1:6 dilution. Cell monolayers were first washed with Hanks' Balanced Salt Solution (HBSS; Life Technologies, Carlsbad, USA), then incubated with trypsin/EDTA (Life Technologies, Carlsbad, USA). Detached cells were resuspended in medium and transferred to new tissue culture flasks.

2.2.1.6 Creation of cell banks

Cells were grown to 70-90% confluence and then trypsinised. Next, cells were counted and centrifuged at 250 *g* for 5 min and subsequently resuspended in freezing medium (90% (v/v) DMEM-based medium and 10% (v/v) dimethyl sulfoxide (DMSO)) at a concentration of 3×10^6 cells mL^{-1} and transferred into 1 mL sterile cryovials. Cryovials were loaded into a Nalgene Mr Frosty freezing container (Sigma, Poole, UK) and placed in a -80˚C freezer. After approximately 24 hours, the cryovials were placed in liquid nitrogen storage.

2.2.1.7 Revival of cells from liquid nitrogen storage

Cells were removed from liquid nitrogen and thawed in a 37˚C water bath. The cell suspension was transferred into a 25 cm^2 tissue culture flask containing 3 mL of pre-warmed DMEM-based medium. Cells were incubated at 37˚C and 10% CO_2 for 2-4 hours to allow cell attachment and the medium was then replaced with fresh DMEM-based medium. The medium was again replaced the following day, and cells were subcultured once 80-90% confluent.

2.2.1.8 Transient transfection

One day prior to transfection, 57R10E cells were seeded into 6-well plates (Nunc, Roskilde, Denmark) at a cell density of 2×10^5 cells mL^{-1} and a well volume of 2 mL. Culture medium was exchanged for 1 mL of fresh medium immediately prior to transfection. Cells were transiently transfected with 0.2 μg per well SIN pHV vector plasmid (shown in Figure 2.1) using FuGENE, a synthethic cationic lipid vector (Promega, Southampton, UK). 24 hours following transfection, the culture medium was exchanged for 1.5 mL of fresh medium. Virus harvest was carried out 48 hours following transfection using a 0.45 μm pore size syringe filter (Minisart® high flow syringe filter, polyethersulfone (PES) membrane, Sartorius Stedim Biotech, Surrey, UK). Clarified supernatant was stored at -80˚C prior to titration.

2.2.1.9 Serum reduction and suspension adaptation

The adaptation to growth at reduced serum concentration was carried out over five passages and is schematically outlined in Figure 2.2. The amount of serum-containing medium was reduced at each passage and replaced with FreeStyle™ 293 Expression Medium (Life Technologies, Carlsbad, USA). Early-passage cells were seeded at a density of 4×10^5 cells mL^{-1} in triplicate 25 cm^2 tissue culture flasks; in addition, one flask of cells growing in 100% DMEM-based medium was maintained as a reference. Cell counts were carried out at each passage. For the packaging cell line, 57R10E, cells were also seeded in 6-well plates and transiently transfected to assess productivity. In case of the producer cell line, EP1, a supernatant sample was taken, syringe filtered and stored at -80˚C prior to titration.

Complete elimination of serum led to severely reduced cell growth, thus 1% (v/v) serum was maintained for cells that were put into suspension. The protocol for

suspension adaptation was that of Griffiths (2000). Briefly, cells were seeded at a concentration of approximately 5×10^5 cells mL^{-1} in 125 mL shake flasks (Corning polycarbonate Erlenmeyer flasks fitted with vent caps; Sigma, Poole, UK) with a working volume of 20 mL. Flasks were shaken on an orbital shaker (IKA KS 260 control, Fisher Scientific, Loughborough, UK) with a shaking speed of 120 rpm. Cell counts were carried out once daily with a sample volume of 0.7 mL. Every three days the medium was removed from the culture, centrifuged at 250 *g* for 5 min and resuspended in fresh medium at a density of at least 2.5×10^5 cells mL^{-1}. As a high level of cell clumping was typically observed, prior to resuspension in fresh medium, the protocol by Griffiths (2000) for minimising cell clumping was followed. This involved removing the cells from the shake flasks, centrifuging at 250 *g* for 5 min and resuspending the cells in 0.01% (v/v) trypsin/EDTA in DMEM (without any other additives). This solution was then transferred back into the shake flasks and incubated at 37˚C for 30 min with a shaker speed of 120 rpm.

2.2.1.10 Growth curves

Cells were seeded at a density of 5×10^5 cells mL^{-1} in triplicate 6-well plates with an initial fill volume of 2 mL. A sacrificial well approach was taken in order to enable cell counts of the adherently growing cells and samples were taken daily.

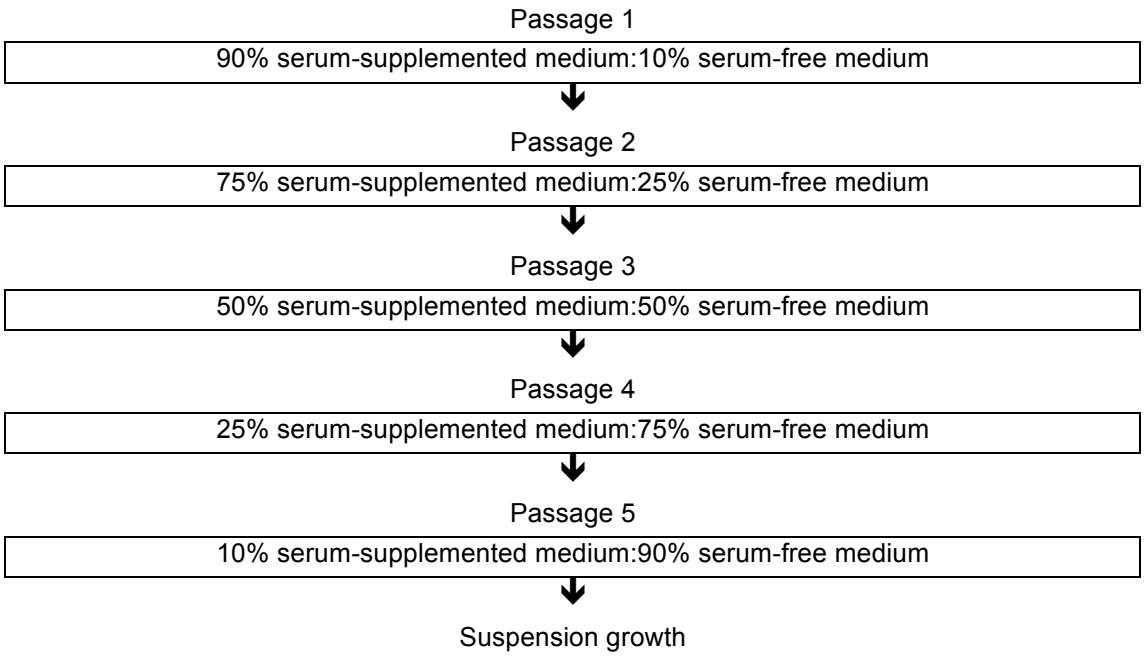


Figure 2.2 Flow diagram illustrating growth medium serum reduction and suspension adaptation strategy.

2.2.2 Vector harvest

Membrane filtration studies were carried out using a syringe pump (Harvard Apparatus, Holliston, USA) set at a constant flow rate dependent on filter area. The flow rate was set so as to maintain a flux of 367 L m^{-2} h^{-1} (LMH) for all filters evaluated. CCF was loaded in a 50 mL syringe along with a magnetic mixing wheel (Rapid Design and Fabrication Facility, UCL), which was rotated with a standard motor to keep solids in suspension. Membranes screened are listed in Table 2.1. Membrane diameter was not constant, therefore, to increase comparability, a constant ratio of feed volume to membrane area of 5 mL cm^{-2} was used, with actual volumes filtered listed in Table 2.1.

Harvest using a Stericup device (Merck Millipore, Darmstadt, Germany) was carried out with a vacuum pump as per the manufacturer's instructions.

All clarification experiments were carried out in duplicate.

Membrane pore size (µm)	Membrane material	Membrane diameter (mm)	Membrane area (cm^2)	Feed volume (mL)	Trade name	Manufacturer
0.45	PES	28	6.2	31	Minisart® high flow Syringe Filter	Sartorius Stedim
0.45	Regenerated cellulose	25	4.9	25	Minisart® RC25 Syringe Filter	Sartorius Stedim
0.45	Polyvinylidene fluoride (PVDF)	33	8.6	43	Millex®-HV Syringe Filter Unit	Merck Millipore
0.22	PES	28	6.2	31	Minisart® high flow Syringe Filter	Sartorius Stedim
0.2	Regenerated cellulose	25	4.9	25	Minisart® RC25 Syringe Filter	Sartorius Stedim
0.22	PVDF	33	8.6	43	Millex®-GV Syringe Filter Unit	Merck Millipore

Table 2.1 Membranes screened for clarification study

2.2.3 Vector concentration and V_{max} analysis

Concentration was carried out using a centrifugal filtration device (100 or 300 kDa molecular weight cut-off (MWCO), PES membrane, Vivaspin 20, Sartorius Stedim Biotech, Surrey, UK). Vector suspensions were centrifuged (5180R, Eppendorf, Cambridge, UK) at 3,000 *g* and 4°C for the time required to reach the desired concentration factor (never more than 20-fold concentration per centrifugation). Concentrations were carried out in triplicate.

For V_{max} analysis, filtration time over cumulative filtrate volume was plotted against filtration time. According to the linearised form of the pore constriction model (Zydney and Ho, 2002) (Equation 2.1) the inverse of the slope on such a plot gives V_{max}.

$$\frac{t}{V} = \frac{1}{Q_0} + \left(\frac{1}{V_{\max}}\right)t, \tag{2.1}$$

where t is filtration time (min), V is cumulative filtrate volume (mL), Q_0 is the initial filtrate flow rate (mL min^{-1}) and V_{max} is the maximum volume that can be filtered before the membrane becomes completely plugged by foulant (mL).

2.2.4 Analytical techniques

2.2.4.1 Cell count and viability

Cell number and viability were determined using a Vi-CELL XR (Beckman Coulter, Fullerton, USA) automated trypan blue dye exclusion method viability analyser, with samples diluted using PBS as necessary.

2.2.4.2 Quantification of lactate and glucose concentration

Lactate and glucose were measured using a Nova BioProfile Analyzer (Nova Biomedical, Waltham, USA).

2.2.4.3 Viral titre assay

Viral titre was determined using a flow cytometry-based gene transfer assay (GTA). HEK 293FT cells were cultured in DMEM-based medium, trypsinised and plated in 24-well plates (Nunc, Roskilde, Denmark) at a density of 5×10^4 cells mL^{-1} with an initial fill volume of 1 mL. Cells were left to attach overnight; the following day two wells were sacrificed to allow cell counts to be carried out prior to exposure to virus.

Polybrene (hexadimethrine bromide (Sigma, Poole, UK)), a cationic polymer, was added to wells to achieve a final concentration of 8 μg mL^{-1}. Polybrene is a transduction enhancer that acts by reducing electrostatic repulsion between cells and vector, as well as contributing to virus aggregation, resulting in more efficient mass transport than if single particles were diffusing to the cell surface (Davis et al., 2004). Vector stock was serially diluted 1:4 twice and 250 μL of each dilution was used to transduce cells, i.e., one well was exposed to 250 μL of virus-containing supernatant, another well to 62.5 μL and a final well to 15.6 μL. The medium was removed 48 h after transduction and cells were washed with HBSS, trypsinised and resuspended in medium. All samples were analysed on a Coulter Epics XL-MCL Flow Cytometer (Beckman Coulter, Brea, USA). 5,000 to 10,000 events were collected and viral titres were calculated from virus dilutions where 5-20% of the cell population was eGFP-positive. Viral titre was calculated as follows: Viral titre (TU mL^{-1}) = ((%eGFP-positive cells) × (number of cells at time of exposure) × (dilution factor)) / (sample volume).

2.3 Results and discussion

2.3.1 Cell growth characterisation

The packaging (57R10E) and producer (EP1) cell lines used in this work were derived recently and their growth has not been fully characterised. Cell growth curves were therefore generated for the null (293FT), packaging (57R10E) and producer (EP1) cell lines.

Cell growth curves for adherently growing cells, cultured in standard medium (DMEM with 10% FBS), were established to allow comparison of cell growth following modifications to the culture conditions, such as suspension culture and serum reduction.

Figure 2.3 (a) shows the viable cell density over an 11-day culture period for the 293FT and 57R10E cell lines, whereas the EP1 cell line was only cultured for six days. Based on cell growth during routine maintenance it was anticipated that the producer cell line would display poor cell growth compared to the packaging cell line, however, from these results it is clear that this was not the case.

57R10E and EP1 are both based on the 293FT cell line. By comparing growth of 293FT to the 57R10E and EP1 cell lines, the impact of virus production on cell growth could be assessed. 293F has a shorter lag phase and grows to a higher cell density than

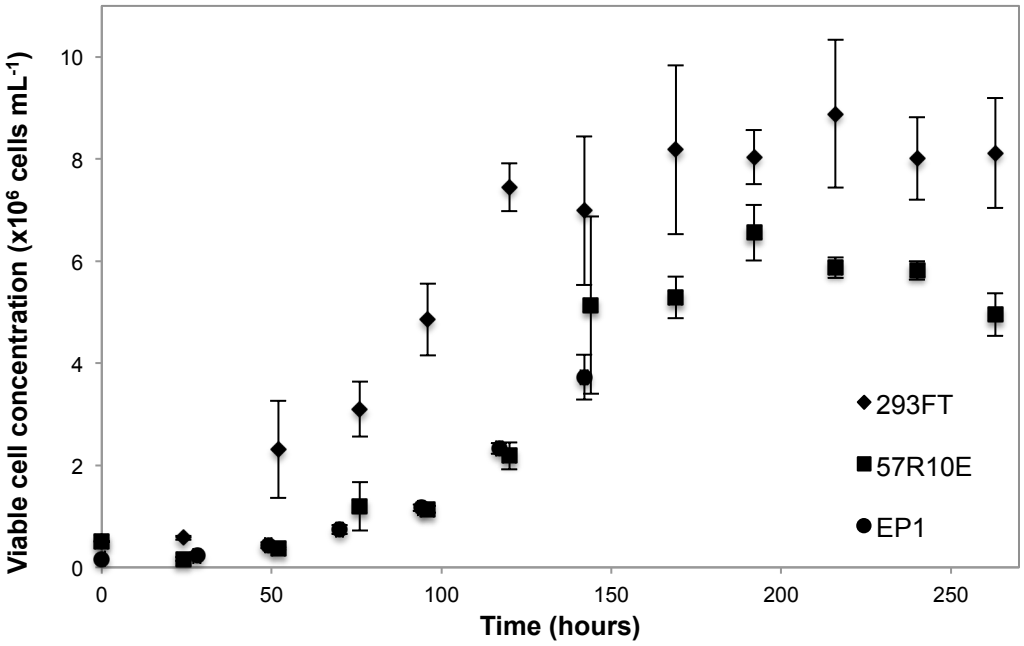
57R10E and EP1. This is to be expected due to the stress of viral component production and virus budding experienced by these two cell lines. As discussed in Section 1.7.2, the Rev protein has been shown to prevent or slow progression through mitosis (Miyazaki et al., 1995). This could explain both the extended lag phase and the lower cell densities achieved with 57R10E and EP1. In addition, the cytotoxic effects of the HIV protease are likely to also have an impact on cell growth.

Large error bars are observed for the null cell line after approximately six days (142 hours) of culture. At this point the cells were confluent (based on qualitative visual observation) and were detaching from the wells during the wash stage, prior to trypsinisation. Thus some viable cells were not counted, and this appears to have happened to a varying degree in the replicate wells, resulting in variable cell counts.

As shown in Figure 2.3 (b), cell viability remains relatively high for all three cell lines during the entirety of the culture periods. However, as spent medium was removed and the cells washed prior to carrying out cell counts, it would be expected that the majority of dead cells were not counted. For adherent cells, it would be beneficial to carry out cell counts of the spent medium and wash solution to determine a more accurate cell count and viability.

Culture medium glucose and lactate concentrations for 293FT and 57R10E are shown in Figure 2.4. As the culture medium was replaced every two days, the characteristic depletion of glucose and accumulation of lactate observed for batch and fed-batch cultivations does not occur (e.g., Paoli et al., 2010; Tsao et al., 2005). Glucose and lactate was measured daily before medium exchange, thus there was always at least one day elapsing between medium exchange and sampling. The cyclical pattern that would have been expected if samples had been taken immediately following medium exchange is therefore not observed. Following the initial decrease in glucose concentration, both cell lines remain at a similar and constant level, except for a reduction in glucose consumption when the cells go into the stationary phase, following 120 hours (five days) in culture.

(a)



(b)

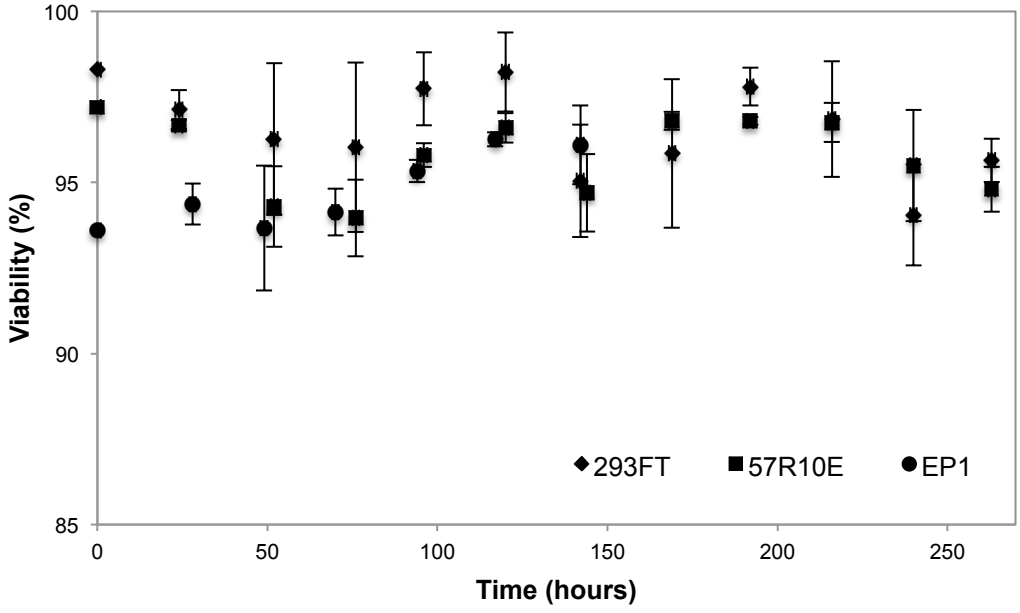


Figure 2.3 Batch replacement cultivation for three different adherent-dependent cell lines grown in 6-well plates. (a) Culture kinetics with medium exchange every two days and (b) cell viability: *(filled diamond)* null cell line (293FT); *(filled square)* packaging cell line (57R10E); *(filled circle)* producer cell line (EP1). Experiments were performed at 37°C with 10% CO_2. Error bars represent one standard deviation about the mean (n = 3).

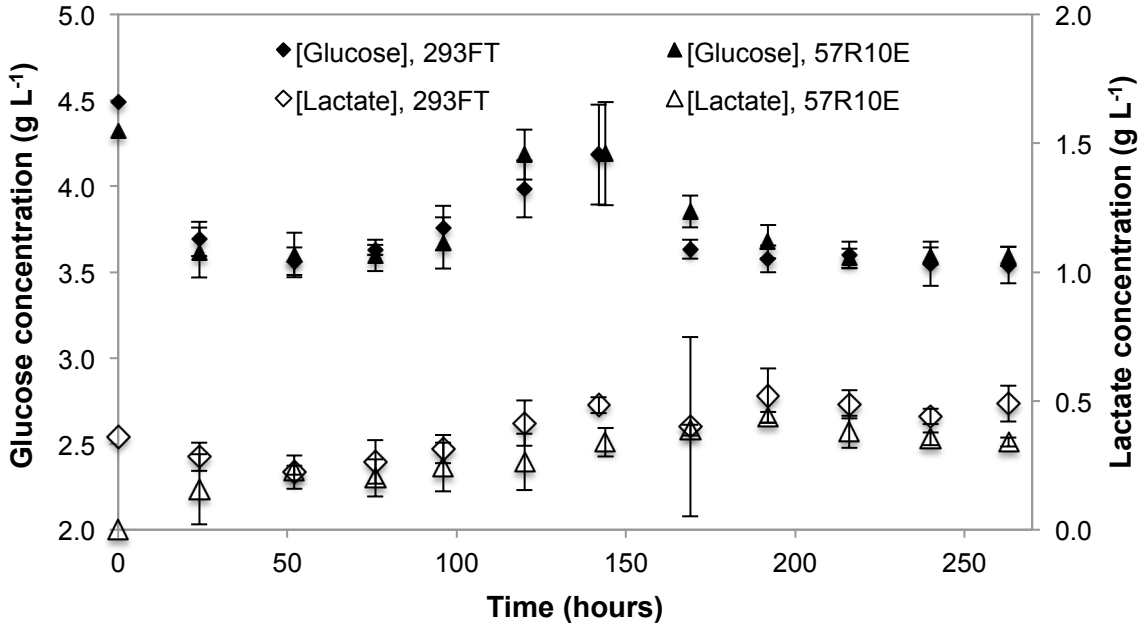


Figure 2.4 The effect of culture duration on glucose and lactate concentration for the null and packaging cell lines: *(filled diamond)* null cell line (293FT); *(filled square)* packaging cell line (57R10E); *(empty diamond)* null cell line (293FT); *(empty square)* packaging cell line (57R10E). Culture conditions and error bars are as in Figure 2.3.

2.3.2 Medium serum concentration reduction

Animal blood serum has been used in mammalian cell culture for over a century (Whitford, 2005). It is added to lean, classical medium formulations to provide cultured cells with the necessary proteins, growth factors, hormones, lipids, vitamins, attachment factors and trace metals. However, serum is also the main source of impurities in harvested LV-containing cell culture material (Segura et al., 2007). As discussed in Section 1.9, there are also a number of other reasons for eliminating the use of serum, including product safety. Schoofs et al. (1998) started by adapting HEK 293 cells to suspension growth, followed by selection of a suitable serum-free medium. The cells used by Schoofs et al. were utilised for adenoviral vector, rather than LV, production. For the work presented here it was decided to follow the recommendation of a group working with LV-producing HEK 293 cells and start with serum reduction (Oxford BioMedica, Oxford, UK, personal communication).

The adaptation to reduced medium serum concentration was carried out over five passages, with a change in serum concentration at each passage. This is in contrast to

Tsao et al. (2001), who used three serum concentration levels and allowed the cells to adapt at each medium composition for 2-3 passages. However, in their study adenoviral vector was produced by infection of HEK 293 cells, thus plasmid retention did not have to be considered. In this work, it was desirable to carry out the adaptation process as rapidly as possible to retain the plasmids for virus production. DMEM supplemented with 10% (v/v) FBS was gradually replaced with FreeStyle™ 293 Expression Medium, which is a chemically-defined, protein-free medium specifically developed for the ability to support the growth of HEK 293 cells under suspension-type culture conditions. The serum concentration was reduced from the initial 10% to, sequentially, 9, 7.5, 5, 2.5 and finally 1%. Complete elimination of serum was attempted, but led to cell death and detachment from the culture surface. Up until a serum concentration of 2.5%, the null cell line grew to the 80-90% confluency-level used for passaging within the normal two day-period (Table 2.2). For the following passage at 1% serum, the cells required three days before reaching confluency, however, a significantly higher viable cell density was achieved, possibly indicating that the cells were adapting well to the low serum concentration.

Table 2.3 shows the results of reduced serum adaptation for the packaging cell line. During routine passaging this cell line displays slower growth than the null cell line, and often requires three rather than two days in-between passages. The higher number of passages requiring three days compared to the null cell line during the adaptation process does therefore not necessarily represent slower growth due to the reduced serum concentration. During the adaptation process, the average cell growth per day remains relatively constant, indicating that cell growth is not dependent on a high serum concentration.

To assess productivity, for passages with 7.5%, 2.5% and 1.0% serum, 57R10E cells were seeded in 6-well plates and transfected with the transfer plasmid. Cells were passaged on Mondays, Wednesdays and Fridays, however, if cell density was low, passaging was delayed. This was typically the case for the producer cell line (EP1). As shown in Table 2.3, the cells still generate a titre above 10^5 TU mL^{-1}, however, the titre is reduced by almost 50% when going from 7.5% to 1.0% serum.

Passage #	[Serum] (% v/v)	Average cell growth per day (x 10^6 cells mL^{-1} day^{-1})	Days between passaging
1	9.0	0.97 ± 0.06	2
2	7.5	0.76 ± 0.21	2
3	5.0	1.22 ± 0.36	2
4	2.5	0.55 ± 0.22	3
5	1.0	1.44 ± 0.43	3

Table 2.2 Medium serum concentration reduction for the null cell line (HEK 293FT). The error represents one standard deviation about the mean (*n* = 3).

Passage #	[Serum] (% v/v)	Average cell growth per day (x 10^6 cells mL^{-1} day^{-1})	Days between passaging	Viral titre (x 10^5 TU mL^{-1})
1	9.0	0.64 ± 0.06	2	N/A
2	7.5	0.47 ± 0.23	3	5.9
3	5.0	0.58 ± 0.06	3	N/A
4	2.5	0.46 ± 0.03	2	3.2
5	1.0	0.77 ± 0.19	3	3.0

Table 2.3 Medium serum concentration reduction for the packaging cell line (57R10E). The error represents one standard deviation about the mean (*n* = 3).

Passage #	[Serum] (% v/v)	Average cell growth per day (x 10^6 cells mL^{-1} day^{-1})	Days between passaging
0	10	0.61 ± 0.05	4
1	9.0	0.97 ± 0.36	5
2	7.5	0.26 ± 0.10	3
3	5.0	0.31 ± 0.23	5

Table 2.4 Medium serum concentration reduction for the producer cell line (EP1). The error represents one standard deviation about the mean (*n* = 3).

Table 2.4 shows the result of the serum reduction process for the producer cell line. During routine passaging, EP1 was observed to grow slower than 57R10E, which was also the case during serum reduction. The producer cell line behaved very differently to the null and packaging cell line as the serum concentration was reduced. At a serum concentration of 2.5%, almost complete cell death was observed within two days.

2.3.3 Suspension adaptation

Successful clinical translation of LV-mediated gene therapy will require large amounts of vector to be produced in a cost-effective manner. As stated in Section 1.9, a project objective was to adapt the packaging and producer cell lines to suspension growth. This would facilitate scalable culture in STRs, which may be one of the ways to reduce the COGs of LV-based therapies.

The ability of HEK 293 cell lines to adapt to suspension-growth in serum-free medium has been an important reason for the popularity of this cell type (Segura et al., 2007). There have been reports of suspension adaptation of HEK 293 cell lines (e.g., Tsao et al., 2001; Schoofs et al., 1998) and there are also commercially available suspension-cell lines (e.g., the FreeStyle™ 293-F cells from Invitrogen).

The suspension adaptation of 57R10E was attempted over 21 days, which corresponded to six passages. For the first passage, cells growing adherently in 90% FreeStyle™ Medium and 10% DMEM-based medium were trypsinised and seeded in shake flasks at a cell density of approximately 5×10^5 cells mL^{-1}. Every three days the cells were spun down and treated with a trypsin solution as described in Section 2.2.1.6. Cell growth over two days following the initial introduction to suspension culture is shown in Figure 2.5 (Passage 1). Viable cell density can be seen to increase day-to-day. During the first three passages severe cell clumping was observed. However, one day following the fourth passage, the cells were no longer visibly clumping, and under the microscope most cells were seen as single cells, with some two-cell aggregates (Figure 2.6 (b)). The large error bars for passage 4 are due to one flask showing close to no increase in cell number over the three days. In addition, as flasks were not seeded at the same cell density at the start of each passage, by this point differences were beginning to accumulate. It was hoped that the absence of large cell aggregates meant that the suspension adaption had been successful, however, during the following passage, cells

again began to clump and a decrease in cell number was observed after two days in culture. After three days, very few cells remained and during passage 6 cell numbers continued to decrease (data not shown). It was then decided to discontinue the adaptation process.

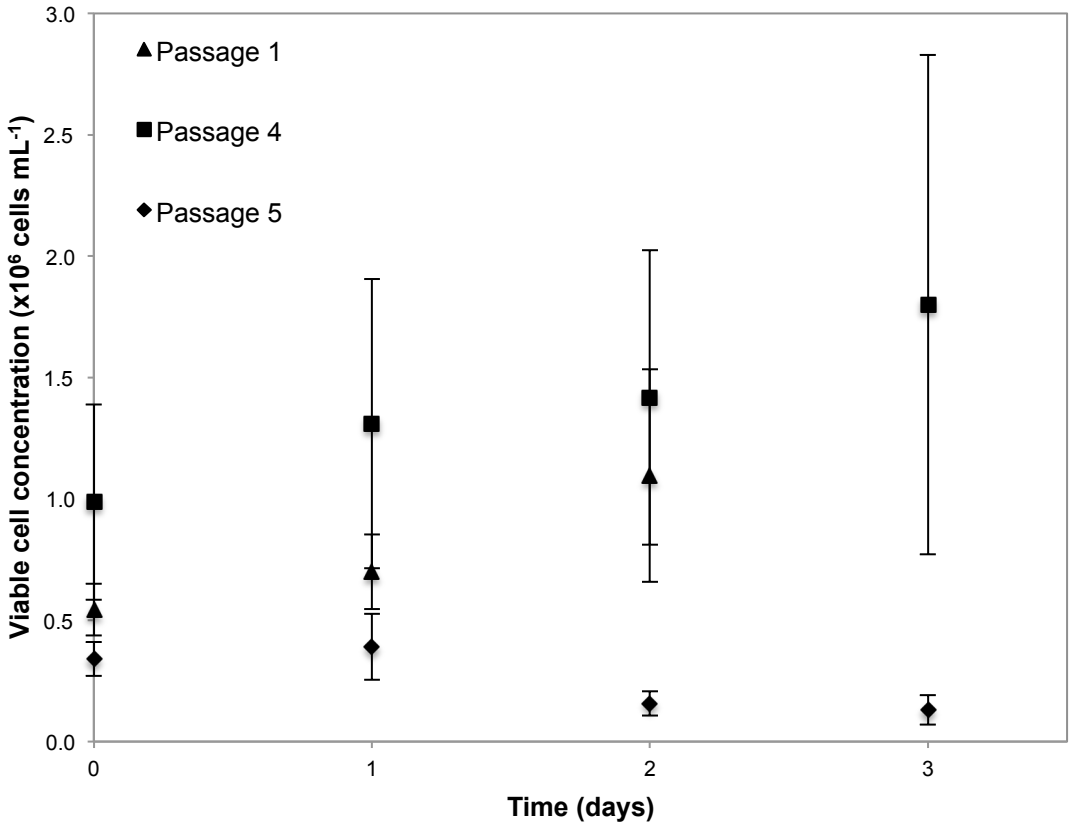
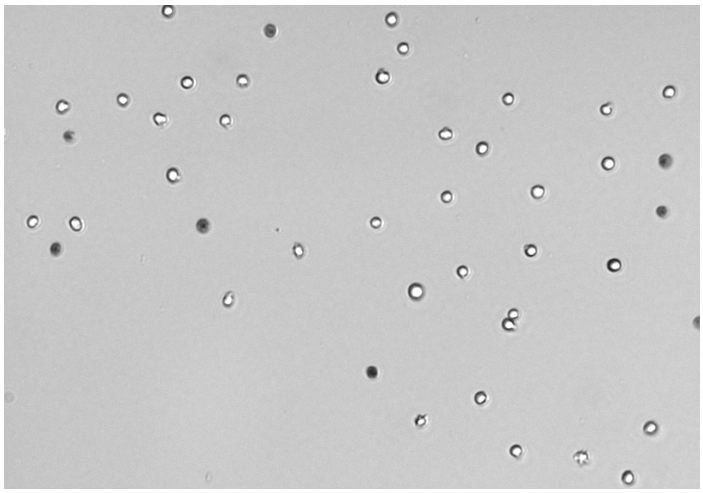


Figure 2.5 Cell growth for representative passages during suspension adaptation of the packaging cell line, 57R10E: *(filled triangle)* passage 1; *(filled square)* passage 4; *(filled diamond)* passage 5. Culture conditions and error bars are as in Figure 3.1.

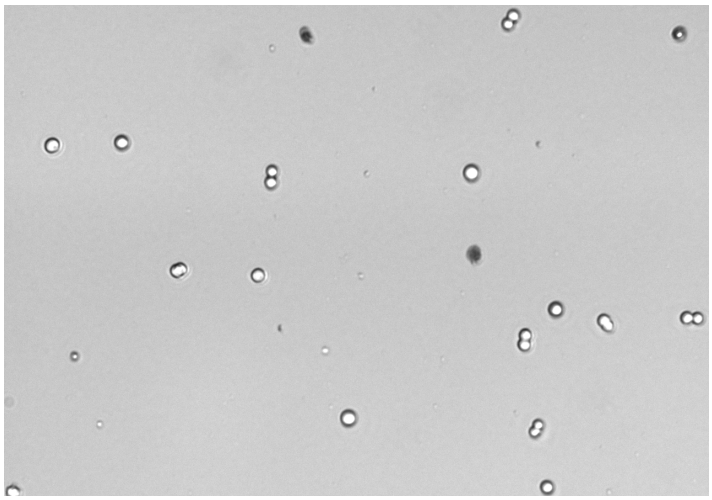
Figure 2.6 shows images of a successfully suspension adapted CHO cell line and 57R10E at two different stages during the adaptation process. Figure 2.6 (b) shows 57R10E cells from passage 4, where the cells were seemingly adapting to the new growth conditions. The cells appear spherical and look very similar to the CHO cell line. Cell aggregates are composed of no more than two cells, and two-cell aggregates can also be seen in the CHO image. However, in the later passage 6, cell growth has stagnated and the cells have lost their spherical shape.

It should be noted that an attempt at adapting the null cell line to suspension growth was made. However, cell clumping was more severe than for the packaging cell line. The more rapid cell growth to higher cell densities exhibited by the null cell line may be a possible explanation for this. A suspension-adapted version of the null cell line would merely have been used as a comparison to the packaging and producer cell lines, therefore adaptation was discontinued.

(a)



(b)



(c)

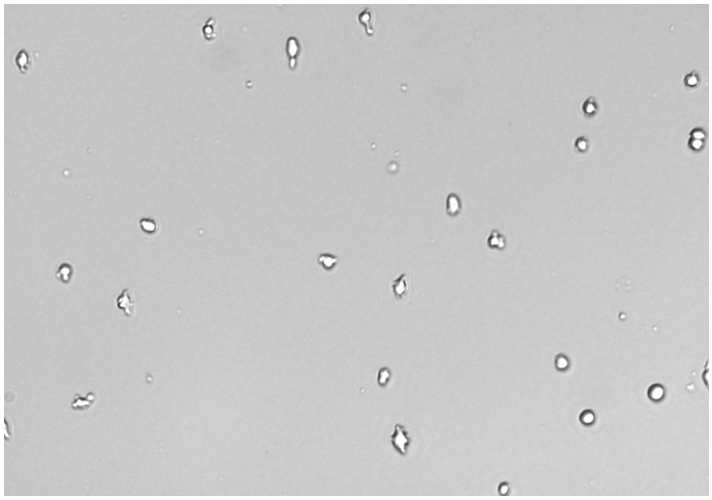


Figure 2.6 Comparison of successfully suspension adapted CHO cell line to packaging cell line grown in suspension. (a) A mAb-producing CHO cell line, (b) 57R10E at passage 4 and (c) 57R10E at passage 6. Images were taken by a Vi-CELL cell viability analyser and experiments were performed at 37°C with 10% CO_2.

2.3.4 Comparison of vector production in culture medium with or without phenol red

Following the suspension adaptation work, it was decided to progress work with the adherently growing STAR-RDpro cell line as the priority was to start with representative LV titres for future downstream purification studies, rather than attempt to develop a transient transfection protocol for a suspension cell line. Due to the low titres ($<10^5$ TU mL^{-1}) achieved with the EP1 cell line (Knight, 2012), STAR-RDpro was the preferred choice as this cell line produces an LV titre of 10^6-10^7 TU mL^{-1} (Ikeda et al., 2003). EP1 was developed in a manner to allow compliance with good GMP, whereas STAR-RDpro was developed with an untraceable cell line and expresses a second generation LV, rather than a third generation, SIN LV as with EP1. Initially, the preferred choice was to work with a traceable cell line, hence STAR-RDpro was not included for suspension adaptation.

STAR-RDpro has historically been cultured in DMEM with phenol red as a pH indicator (Ikeda et al., 2003). An objective for this thesis involves the use of anion exchange chromatography as a LV capture step. Unfortunately, as phenol red binds irreversibly to anion exchange chromatography resins at pH > 7 (GE Healthcare), it would be beneficial to evaluate a phenol red-free medium. The most similar off-the-shelf medium was selected and supplemented with sodium pyruvate and additional L-glutamine, as well as FBS and antibiotics. The infectious titre obtained 48 h following cell seeding in both mediums is shown in Figure 2.7. The average titre is lower in the phenol red-free medium, however, this is statistically insignificant for $p < 0.05$ (*t*-test, $p = 0.20$) and the titre is sufficiently good to produce useful amounts of vector for chromatography and formulation work.

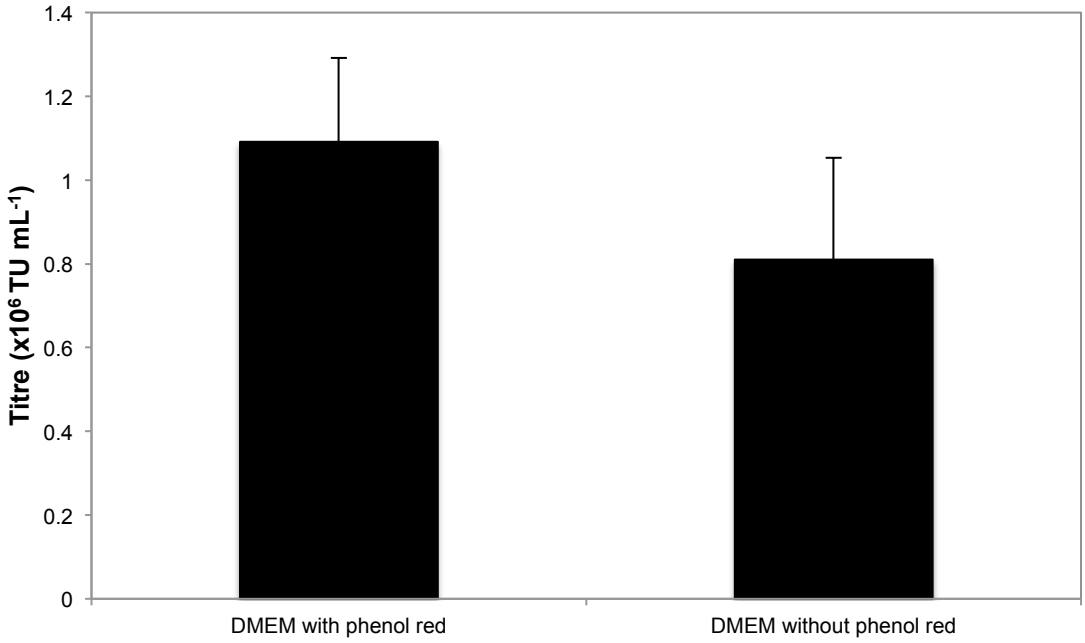


Figure 2.7 Infectious titre in historically used culture medium containing phenol red compared to culture medium without phenol red. Cells were grown in 25 cm^2 tissue culture flasks at 37°C with 10% CO_2. Error bars represent one standard deviation about the mean (n = 3).

2.3.5 Clarification by membrane filtration of RDpro-pseudotyped HIV-1 lentiviral vector

CCF from adherently growing cells has a significantly lower solids concentration than CCF from suspension cells, however, clarification is still necessary to remove dead cells and cell debris prior to chromatography. Three commonly used membrane materials (regenerated cellulose, PES and PVDF), in two different pore sizes (0.22 and 0.45 μm), were evaluated, as shown in Figure 2.8. No statistically significant difference in vector recovery is observed between membrane material (t-test, $p < 0.05$) (please see Appendix 1 for p-values). Pore size, however, has a large and statistically significant impact on recovery (t-test, $p = 3.8 \times 10^{-9}$). The vector particles are approximately 100 nm in diameter – less than half the size of the 0.22 μm membrane pores. It is likely that membrane fouling from cells and cell debris lead to an effectively smaller pore size, resulting in reduced product transmission. Harvesting of clinical material is typically carried out with a depth pre-filter (retention rating > 1 μm) (sometimes omitted) followed by a series of membranes of decreasing pore size (typically 0.8 μm followed by 0.45 μm) (e.g., Biffi et al., 2013; Merten et al., 2010; Slepushkin et al., 2003). These clinical processes omit a 0.22 μm membrane step for clarification and in the absence of

published results, perhaps it can be inferred that low recoveries following 0.22 μm filtration are observed across the field. Due to the poor recoveries with 0.22 μm pore size membranes, some groups have gone so far as to emit sterile filtration from the end of the process (Ausubel et al., 2012). Oxford Biomedica has a patent that covers concentration following sterile filtration, thereby challenging the industry convention of always carrying out sterile filtration as the last operation before formulation (Truran et al., 2009). It should be possible to improve sterile filtration recoveries by optimising filtration parameters, such as the feed volume to membrane area ratio used.

An average recovery of infectious particles above 100% is observed for the PES and PVDF membranes. This is an artefact of the cell-based assay used to determine viral titre. The cells and cell debris present in the samples taken before clarification may act as transduction inhibitors, thereby artificially reducing the functional titre observed before clarification. A similar effect has been reported for ultrafiltration of γ-retroviral vectors, where yields higher than 100% were obtained when a 300 kDa MWCO membrane was used (Le Doux et al., 1996; Segura et al., 2005). In the case of ultrafiltration, the removal of proteoglycans and small DNA fragments that could reduce the transduction enhancing benefits of polybrene were deemed likely explanations for the above 100% yield.

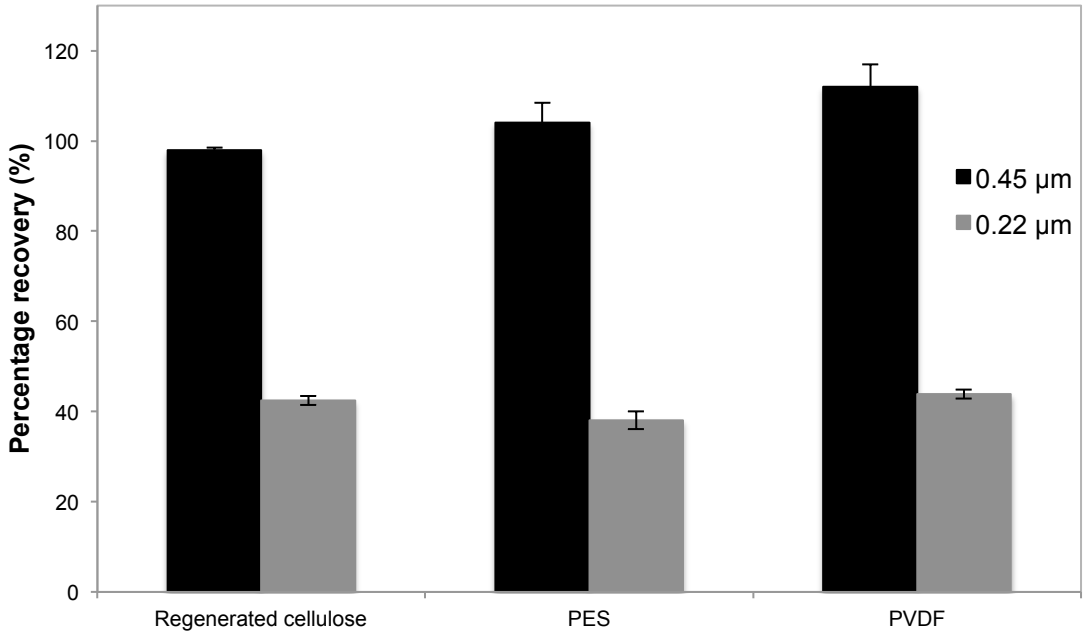


Figure 2.8 Recovery of infectious particles following syringe pump-driven, normal flow filtration using three different membrane materials (regenerated cellulose, PES and PVDF) and two different membrane pore sizes (0.22 µm and 0.45 µm). Filter size varied and therefore a feed volume to membrane area ratio of 5 mL cm^{-2} was used for all filters to enhance comparability. A constant flux of 367 LMH was used for all filters and was achieved by varying the flow rate delivered by the syringe pump used for loading. Solids were kept in suspension by the use of a magnetically rotated impeller in the syringe used to hold the feed material. Values shown are the mean of two experiments and error bars show the actual data points.

Syringe filtration is only practical for small feed volumes and for this reason the use of a Stericup with a 0.45 µm pore size was compared to syringe filtration with respect to recovery of infectious vector particles (Figure 2.9). It is desirable to keep the feed stream sterile as a cell-based assay is used to quantify infectious titre.

The average functional vector recovery following Stericup filtration was lower (86%) than for syringe filtration (97%). This result was statistically significant for $p < 0.05$ (t-test, $p = 0.02$). The Stericup recovery, however, was high enough that sufficient amounts of vector would remain for studies further downstream.

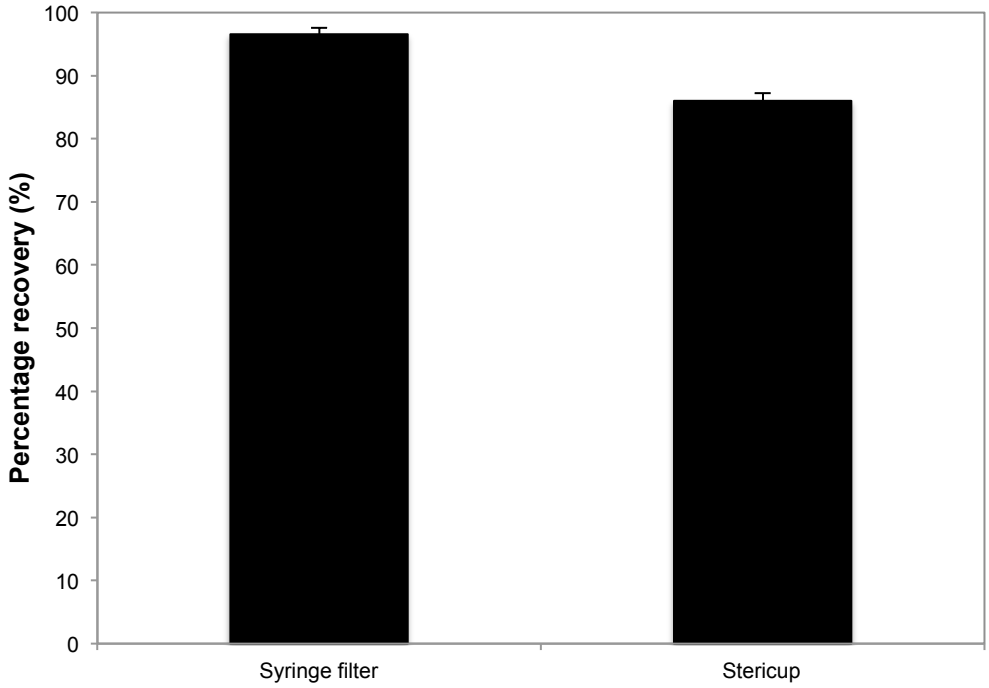


Figure 2.9 Recovery of infectious LV particles following normal flow filtration using either a syringe-driven filtration unit or a vacuum-driven Stericup. The syringe filter had a PES membrane whereas the Stericup contains a PVDF membrane. Both devices had a 0.45 µm pore size. A feed volume to membrane area ratio of 5 mL cm^{-2} was maintained for both filtration methods. Values shown are the mean of two experiments and error bars show the actual data points.

2.3.6 Concentration of RDpro-pseudotyped HIV-1 lentiviral vector

Chromatography binding studies, described in Chapter 3, and formulation studies, described in Chapter 4, require feedstock to be prepared in different concentrations and in a variety of buffers. Centrifugal filters were evaluated as a simple concentration method as they require no specialist equipment and have a minimal hold-up volume. Centrifugal filters have been used successfully for the concentration of LVs, both for research and pre-clinical/clinical application purposes, and require only a standard laboratory centrifuge, unlike concentration by ultracentrifugation (Strang et al., 2004; Zimmerman et al., 2011; Benati, 2012). Like other retroviruses, HIV-1 has a mass of $\sim 2.5 \times 10^5$ kDa (Vogt and Simon, 1999), which enables the use of large-pore size ultrafiltration membranes, thereby reducing the processing time required to achieve a set concentration factor and enabling the removal of relatively high molecular weight impurities. Membranes with MWCO of 100 and 300 kDa have been commonly used for

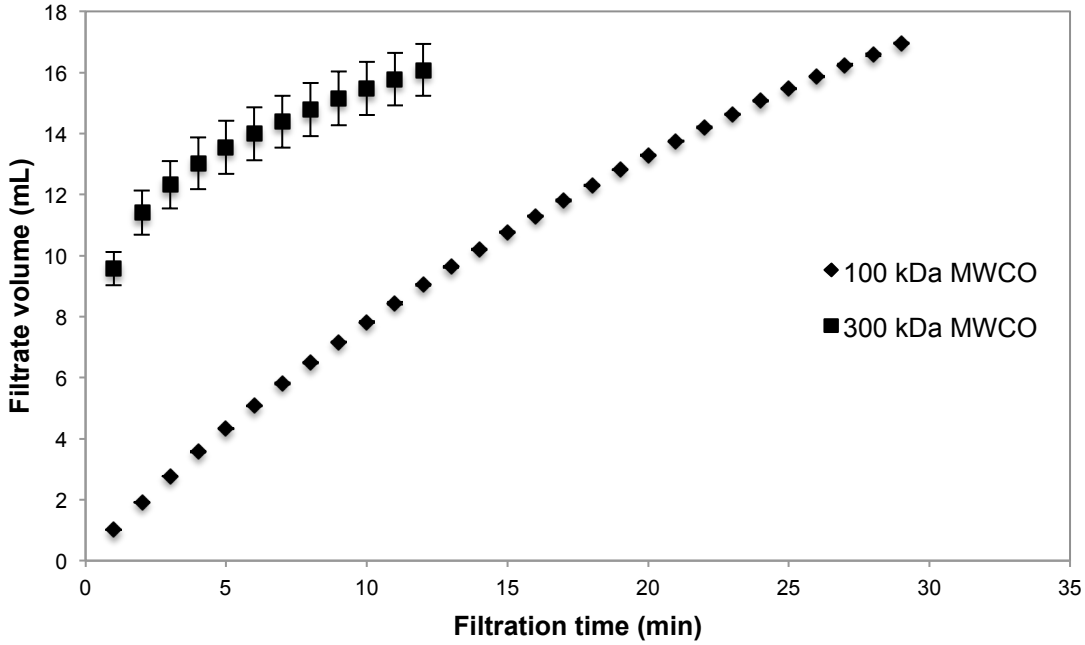
concentration of γ-retroviral and LVs (e.g., Segura et al., 2005; Strang et al., 2004; Transfiguracion et al., 2003).

Cell culture supernatant from the STAR-RDpro producer cell line was clarified and 20 mL of this material was added to a centrifugal filtration device. Devices with 100 and 300 kDa MWCOs were compared with respect to time taken to achieve a concentration factor (CF) of approximately 10 and recovery of infectious vector particles. In Figure 2.10 (a), the cumulative filtrate volume profiles for the two pore sizes can be seen to be very different. For the 300 kDa membrane, a large volume permeates quickly, but as the membrane becomes fouled, the filtration rate is reduced. The large initial flux leads to a processing time of only 12 min to achieve the desired CF. In contrast, the 100 kDa membrane follows a linear trend initially and the filtration rate declines more slowly than for the 300 kDa membrane after the initial linear region. Compared to the 300 kDa membrane, the concentration time is more than doubled when using the 100 kDa membrane. The additional processing time, however, does not have a negative impact on active vector recovery, as 83% (± 5.4%) of active particles are recovered following concentration using the 100 kDa membrane, whereas only 24% (± 3.0%) are recovered from the 300 kDa membrane (Figure 2.11). The difference in recovery should not be related to pore size as vector particles are sufficiently large that they should not be able to permeate either membrane and no infectious particles were detected in the permeate. Figure 2.10 (a) suggests that membrane fouling occurs rapidly for the 300 kDa unit and this could be related to vector inactivation due to e.g., irreversible entrapment in the gel layer or damage to the vector envelope due to impact with the membrane. Strang et al. (2004) reported a 21% recovery of the same vector as the one used in this study following concentration with a 100 kDa centrifugal filter, however, they concentrated 40-fold and the increased CF would be expected to result in a lower recovery of active particles. Zimmerman et al. (2011) used the same type of device for concentration of a VSV-G pseudotyped LV and also observed a higher recovery using a 100 kDa unit compared to 300 kDa. The difference in their case was less pronounced, which might be a result of the stability of the VSV-G envelope compared to RDpro. It is likely that the high initial flux with the 300 kDa membranes is what leads to product loss. If a filtration system was used where the transmembrane pressure could be increased slowly, it may be possible to use 300 kDa, or larger, pore size membranes and still retain infectivity.

V_{max} analysis is used for sizing normal flow filters that foul according to the pore constriction model (Zydney and Ho, 2002). The filters used in this study are not strictly operated in normal flow mode, but it was hoped that this analysis would at least provide an estimate of V_{max}, the maximum volume that can be filtered before the membrane becomes completely plugged by foulant. For the concentration units used in this work, 20 mL of solution can be filtered per run and V_{max} analysis was used in this case not to size a large-scale filter, but to assess if the concentration devices could be reused. Equation 2.1 shows that taking the inverse of the slope of the linear regression curve in Figure 2.10 (b) will give us V_{max}. The V_{max} value obtained for the 300 kDa unit was 17 mL ($\frac{1}{0.058}$ mL), which is close to the volume that was actually filtered. Towards the end of the filtration time the flux was very low, providing some proof that V_{max} analysis can be useful for this type of centrifugal concentrators. For the 100 kDa unit V_{max} was found to be 42 mL ($\frac{1}{0.024}$ mL), indicating that these membranes can be reused and this was also observed in practice.

For chromatography studies and formulation studies described in Chapters 3 and 4, it would be advantageous to have highly concentrated vector stocks to maximise the dilution factor into buffers. The starting volume (20 mL) of the centrifugal concentrators makes it difficult to go beyond a CF of 40. To enable higher CFs, the retentate from three concentrators (100 kDa MWCO) was pooled and concentrated further. An overall CF of 80 was achieved with a recovery of active vector particles of 65%.

(a)



(b)

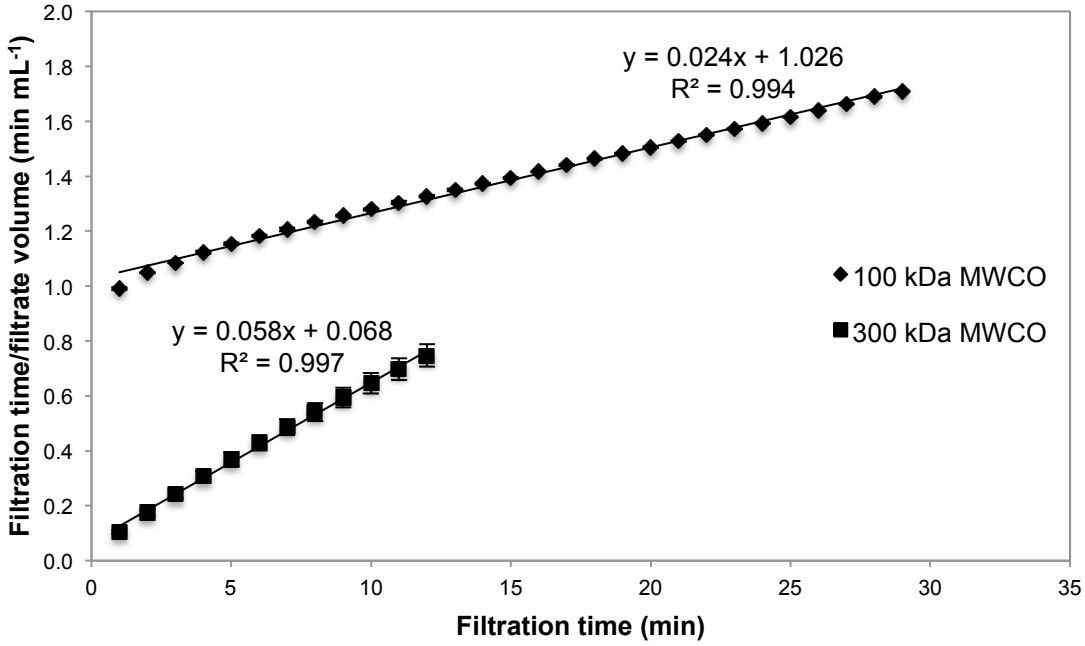


Figure 2.10 Concentration of a HIV-1 vector by centrifugal filtration. Clarified cell culture supernatant was concentrated 10-fold at 3,000 *g* and 4°C using either a 100 or 300 kDa MWCO PES Vivaspin 20 unit. The cumulative filtrate volume as a function of centrifugation time is shown in (a) and (b) shows V_{max} analysis to enable determination of the maximum volume that can be filtered by each unit. Error bars represent one standard deviation around the mean (n = 3).

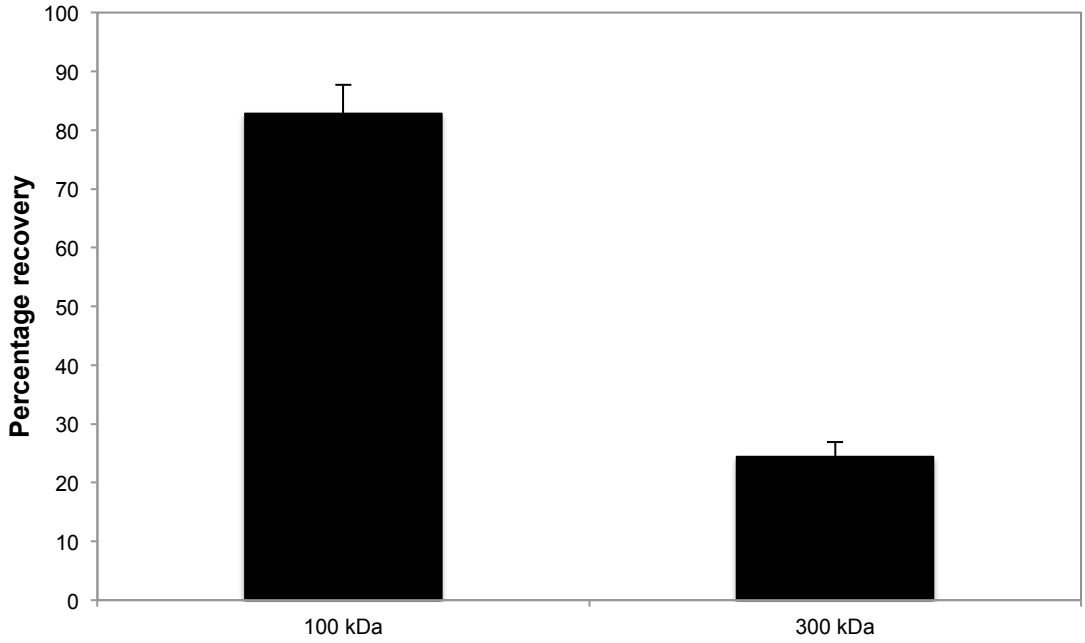


Figure 2.11 HIV-1 vector yield following concentration by centrifugal filtration. Clarified cell culture supernatant was concentrated 10-fold using either a 100 or 300 kDa MWCO PES Vivaspin 20 unit. Samples were titrated in duplicate on HEK 293FT cells and eGFP expression was assayed by flow cytometry. Values shown are the mean of two experiments and error bars show the actual data points.

2.4 Conclusion

Cell growth curves for three anchorage-dependent cell lines based on HEK 293FT were developed. The null cell line was found to grow at a higher exponential growth rate and to a higher viable cell density than the two other cell lines. This may have been due to the large number of extra proteins – some of which are known to be cytotoxic – expressed by the packaging and producer cell lines.

Reduction of the growth medium serum concentration was attempted for the three cell lines. While the null and packaging cell lines tolerated a serum concentration as low as 1% (v/v), it was found that the producer cell line required more than 2.5% (v/v) of FBS to maintain cell growth.

Adaptation to suspension culture has been described for the LV packaging cell line 57R10E. When the packaging cell line was growing steadily at 1% (v/v) serum, culture in shake flasks commenced. Severe cell aggregation was observed, however, cell division, rather than cell death, was dominating and an increase in viable cell density was typically observed day-by-day. Cell clumping was alleviated by incubation

at 37 °C in a dilute trypsin solution for 30 min at each passage. Four passages into the adaptation process, no visible cell clumping was observed and photographs at the cell-level showed aggregates no larger than two cells. However, during the following passage visible aggregation was again occurring and cell numbers were declining. This carried on during the next passage and it was decided to discontinue the adaptation process.

The remainder of work was carried out with the STAR-RDpro cell line. To enable the production of material suitable for AEX chromatography studies, this cell line was adapted to growth in culture medium without phenol red, with no significant reduction in infectious titre.

Membrane material for clarification was found to not have an impact on the recovery of functional vector particles, however, membrane pore size had a large effect. A 0.45 μm pore size led to a recovery of approximately 100% for all membrane materials, whereas a pore size of 0.22 μm resulted in recoveries around 40% for all materials. The use of a Stericup device for clarification led to a 10% reduction in functional vector recovery compared to syringe filtration, however, the recovery was sufficiently high to allow the use of this clarification method for fast preparation of material for further downstream studies.

Vector concentration utilising centrifugal filters was possible with high recoveries (>80%) for filters with a MWCO of 100 kDa, whereas low recoveries (<25%) were seen with a MWCO of 300 kDa. A CF as high as 80, with a functional vector recovery of 65%, was achieved by first concentrating CCF and then pooling the retentate from several filters and repeating concentration.

3. CHROMATOGRAPHIC CAPTURE OF AN RDPRO PSEUDOTYPED LENTIVIRAL VECTOR USING MEMBRANE- AND MONOLITH-BASED STATIONARY PHASES

3.1 Introduction

Chromatography has become the workhorse of most industrial-scale protein separation processes due to its scalability, consistency, fast processing times and amenability to automation. Ion exchange chromatography is the most commonly used method as it offers good impurity removal and the resins are relatively affordable and easy to clean (Trilisky, 2009). Most viruses carry an overall negative charge at neutral pH (Michen and Graule, 2010), which makes purification by AEX chromatography a suitable option. γ-retroviral vectors and subsequently VSV-G-pseudotyped LVs have been purified by AEX chromatography (e.g., Lesch et al., 2011; Merten et al., 2010; Rodrigues et al., 2008; Slepushkin et al., 2003), however, to date, process optimisation efforts for LV purification have been absent or very limited. Optimisation of the conditions used during chromatography is important for LV purification not only to maximise process metrics such as dynamic binding capacity and impurity removal, but most importantly for retention of vector infectivity.

Typically, the stationary phase used for chromatography is particle-based, but due to the large size of viruses, there has been growing interest in the use of monolithic resins and membrane adsorbers for virus bioprocessing (Jungbauer and Hahn, 2008). For particle-based adsorbents, the majority of binding sites are located in small, internal pores, into which mass transfer is limited by diffusion. These pores are typically smaller or in the same size order as the diameter of LVs, thus binding will occur predominantly on the surface of the beads, resulting in poor ligand utilisation. Monolithic adsorbents are highly porous, allowing mass transfer to occur predominantly by convection, which results in retention of dynamic binding capacity and resolution at high flow rates (Barut et al., 2005; Jungbauer and Hahn, 2008; Podgornik et al., 2000). The compatibility of monoliths with high flow rates means that processing times can be kept short, which should enable better retention of LV infectivity. To date, there have been three reports of LV purification using monoliths (Bandeira et al., 2012; Cheeks et al., 2009; Lesch et al., 2011). Recoveries above 60% were achieved in all cases, thereby demonstrating the potential of monolithic resins for LV purification.

LVs differ to traditional biopharmaceuticals, such as mAbs, not only due to their significantly more complex structure, but also because they are typically not the final drug product. LVs are predominantly used to transduce patient cells *ex vivo*, and the final drug product consists of transduced cells that have been processed further following transduction. This means that the purity requirements for formulated LVs are unlikely to be as stringent as for a parenteral biopharmaceutical. Therefore, a LV downstream process platform will most probably not mimic the typical three chromatography step processes seen for mAbs. A well-designed chromatography capture step followed by concentration and formulation by ultrafiltration may well form the backbone of a LV downstream process platform. In the absence of a suitable affinity-based purification, AEX has become the method of choice for LV purification.

Biopharmaceutical process development most logically occurs early during product development when the chance of clinical failure is still high. The resources expended on this stage should therefore be minimised. This approach, however, can lead to less than ideal processes and issues may be encountered upon scale-up to production scale. Experimentation at the microlitre scale would mean that a wider range of process conditions can be studied at a much earlier stage, and may lead to more robust and scaleable processes with lower COGs (Micheletti and Lye, 2006). In addition, automated microscale approaches, when used in conjunction with statistical DoE, are excellent tools for application of the QbD model (Chhatre and Titchener-Hooker, 2009). The QbD paradigm will put further pressure on biopharmaceutical companies to carry out high-throughput experimentation in order to define the process design space (Legmann et al., 2009).

Reports of bioprocess optimisation at the microlitre to millilitre scale, so called ultra scale-down (USD) approaches, have been reported for a large number of unit operations and some recent examples include cell culture optimisation for LV production (Guy et al., 2013), filtration- and centrifugation-based primary recovery (Lau et al., 2013) and cell concentration and buffer exchange by TFF (Masri, 2014). For the development of chromatography processes, a number of commercialised systems are available and include RoboColumns® from Atoll, PreDictor™ plates from GE Healthcare and pipette tip columns from PhyNexus. In this work, monoliths and membrane exchangers were used and these stationary phases have the benefit of not requiring packing and can simply be added to microtitre plates as for filter plates. These high-throughput approaches allow a large number of experiments to be conducted

rapidly, however, there is a real danger of simply moving the experimental bottleneck further downstream to the analysis stage (Konstantinidis et al., 2013). This is especially true for products such as LV that are dependent on complex cell-based assays for meaningful quantification. It is also important to consider that once the experimental analysis bottleneck is solved, there is a risk that the next step, data analysis, becomes a bottleneck. And once that challenge is solved, the next difficulty lies with understanding the results and making decisions based on the data.

The specific aims and objectives of this chapter are:

- to characterise monolith 96-well plates with respect to flow rates through wells and uniformity of dynamic binding capacity across plates;
- to determine binding and elution conditions for an RDpro pseudotyped LV in 96-well monolith and membrane plates; and
- to verify the conditions determined in 96-well plates with 1 mL laboratory scale columns operated by a chromatography system.

3.2 Materials and methods

3.2.1 Feed material

STAR-RDpro producer cells cultured in 175 cm^2 tissue culture flasks or TripleFlasks (culture area = 3 × 175 cm^2) (Nunc, Roskilde, Denmark) were used for vector production. Cells were seeded at a density of 8×10^4 cells cm^{-2} and vector was harvested 48 h following seeding. Depending on harvest volume, harvested material was clarified with syringe filters or a Stericup device (Merck Millipore, Darmstadt, Germany), both with membranes with a 0.45 μm pore size. Due to the deleterious effect of freeze-thawing (approximately a 25% reduction of infectious titre, please see Chapter 4, Section 4.3.1), vector material was always harvested on the day of use.

3.2.2 Chromatography 96-well plates

The high-throughput plates used in this work were Convective Interaction Media (CIM®) 96-well monolithic plates (BIA Separations, Ljubljana, Slovenia), with each well containing 200 μL of AEX resin, and Sartobind® 96-well membrane adsorber plates (Sartorius Stedim Biotech, Surrey, UK), with each well containing 19 μL of AEX resin. The AEX resin in the monolithic plates had either DEAE ligands, a weak anion

exchanger, or Q ligands, a strong anion exchanger, whereas only Q membrane plates were used. The monolith support matrix is based on methacrylate and the membranes are based on stabilised reinforced cellulose.

3.2.3 Robotic operation of chromatography 96-well plates

The 96-well plates were operated by a Tecan Freedom EVO 150 robot (Tecan, Männedorf, Switzerland). The instrument has an 8-tip pipettor arm for liquid handling and uses disposable tips with integrated conductivity probes that allow liquid level detection. A robot-driven Te-VacS vacuum system (Tecan, Männedorf, Switzerland) was used to separate stationary and mobile phases by vacuum filtration at -0.7 barg.

Chromatographic separations consisted of the steps described in Table 3.1. Buffers were held in 100 mL reservoirs and were pipetted using 1,000 µL conductive tips (Biorobotix, VWR International, Lutterworth, UK).

Step	Agent	Volume (CVs)
Remove storage buffer (20% ethanol in water)	Water	10
Equilibration	Same as loading buffer	5
Loading	Please see text	Please see text
Washing	Same as loading buffer	5
Elution	Loading buffer with 1.5 M NaCl or 50 mM Tris, pH 7 with 1.5 M NaCl if loading clarified cell culture supernatant (for elution study, please see text)	10
Regeneration and post-run cleaning	2 M NaCl	10
	Loading buffer	10
	1 M NaOH	5
	Water	10
	Concentrated loading buffer	10
	Water	10
	Loading buffer	10
Storage	20% ethanol	4

Table 3.1 The sequence of steps used for operation of 96-well monolithic plates.

3.2.4 Characterisation of 96-well monolithic plates

3.2.4.1 Determination of well flow rates

The flow rate through the wells was determined by adding 0.95 mL of Milli-Q water (Millipore, Bedford, USA) to a representative selection of wells, shown in Figure 3.1,

and collecting and weighing the flow-through of the wells after 30, 45, 60, 75 and 90 s. The procedure was repeated in triplicate for each time point. Flow rates were obtained by finding a relationship between flow-through volume and time using linear regression.

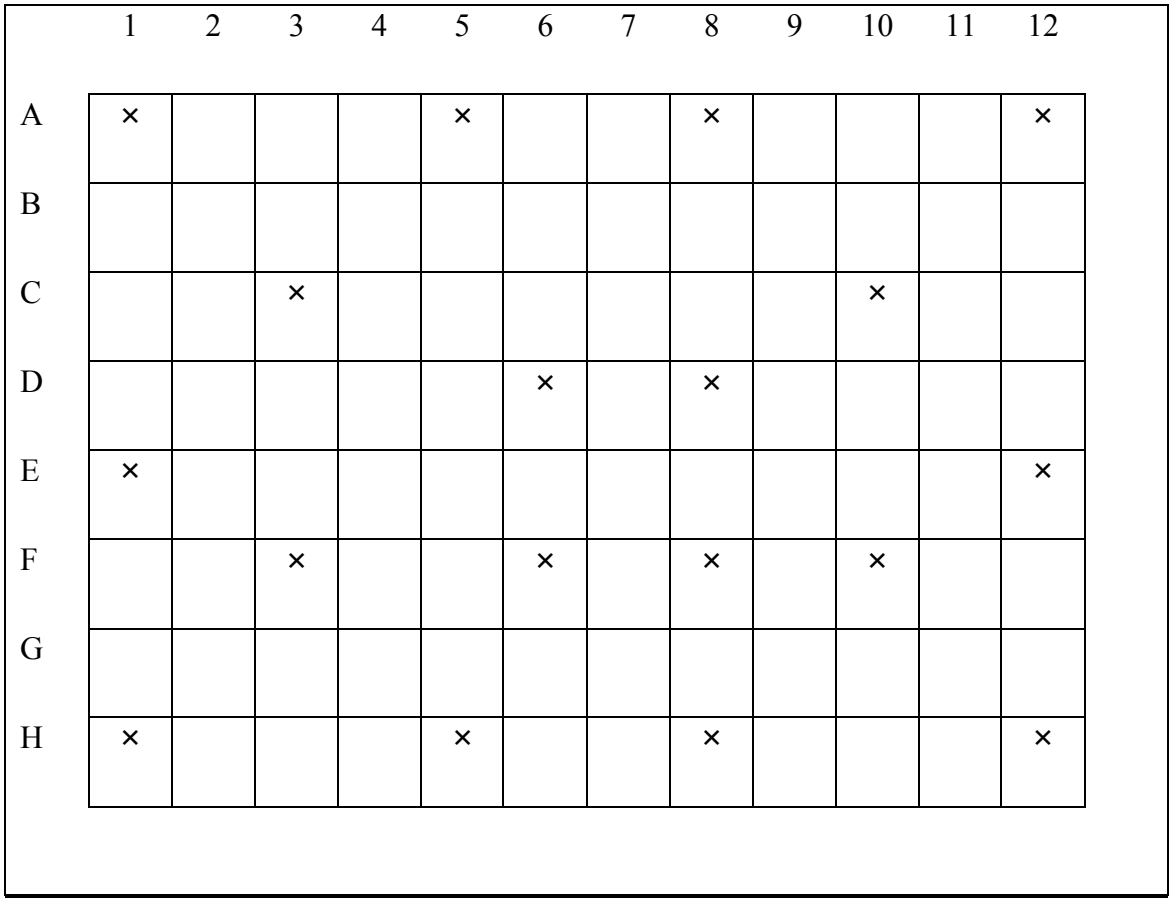


Figure 3.1 The flow rate of water through representative wells (indicated with ×) containing monolithic resin was measured after application of vacuum (-0.7 barg) for 30, 45, 60, 75 and 90 s.

3.2.4.2 Determination of well protein binding capacities

To assess resin protein binding capacity, 1 mL of clarified cell culture supernatant was loaded onto each well of a 96-well monolithic plate containing resin with Q ligands and the total protein concentration of the feed and flow-through, wash and eluate fractions was determined.

Binding capacity was calculated using Equation 3.1:

$$q = \frac{m_{protein,loaded} - m_{protein,remaining}}{V_{resin}}, \tag{3.1}$$

Where q is protein binding capacity (mg mL^{-1}), $m_{protein,\ loaded}$ is the mass of protein added to the resin during the load step (mg), $m_{protein,\ remaining}$ is the mass of protein found in the flow-through fraction (mg) and V_{resin} is the volume of the chromatography resin (0.2 mL).

3.2.5 Investigation of loading conditions

The purpose of this investigation was to determine the binding capacity of the resins under different loading buffer (i.e., the buffer used for feed dilution and which were identical to those used for equilibrating and then washing the matrix after feed application in each experiment) conditions, therefore all experiments were undertaken with a feed volume chosen so as to overload the wells. This work was carried out in 96-well plates containing 200 µL of monolithic resin. The experiment was carried out for one strong (Q) and one weak (DEAE) exchanger. Three buffer species were assessed: Tris/HCl, HEPES and sodium phosphate. LVs are stable at physiological pH (≈ 7.4) and hence buffer pH range was chosen around that value. The pH values are not the same for the buffers as their buffering range is not exactly the same, however, they were chosen as they offer buffering capacity around physiological pH. The choice of buffer concentration was based on obtaining sufficient buffering capacity. For HEPES and phosphate buffers, a buffer concentration of at least 50 mM is recommended for adequate buffering capacity, whereas 20 mM is enough for Tris (Haynes, 2011).

A DoE approach was used to reduce the number of experiments required compared to a OFAT approach. In addition, any interactions between factors can be elucidated with a DoE study. Due to the low number of factors and small pH range studied, a response surface method design was chosen directly as opposed to carrying out an initial screening design. A D-optimal design was chosen to limit the number of runs required. The design was generated using Design Expert version 7 (Stat-Ease Incorporated, Minneapolis, US). One design per buffer was generated and the conditions used can be found in Table 3.2.

Clarified cell culture material was concentrated 10-fold using the Vivaspin 20 (100 kDa MWCO) tubes as described in Section 2.2.3. 200 µL of concentrated vector harvest was added to 1.8 mL of loading buffer.

Tris/HCl		Phosphate		HEPES	
pH	**Buffer concentration**	**pH**	**Buffer concentration**	**pH**	**Buffer concentration**
7.2	36	7.0	20	7.0	20
7.2	36	7.0	20	7.0	20
7.7	20	7.5	20	8.1	20
7.7	20	8.0	46	8.1	20
8.5	20	8.0	46	7.4	30
8.5	20	7.8	33	7.9	33
8.1	32	7.0	35	8.5	33
7.2	36	7.5	37	8.5	33
8.5	40	8.0	46	7.0	43
7.7	42	8.0	47	7.7	43
8.2	50	7.2	60	8.2	47
7.9	56	7.0	60	7.0	60
7.2	60	7.0	60	7.0	60
7.2	60	7.4	60	7.8	60
8.5	60	7.5	60	8.5	60
8.5	60	7.5	60	8.5	60

Table 3.2 Loading buffer conditions evaluated.

3.2.6 Investigation of elution conditions

Elution by increasing NaCl concentration was assessed in 96-well monolithic (Q and DEAE ligands) and membrane (only Q ligand) plates. Elution buffers consisting of 20 mM Tris/HCl, pH 7.4 with NaCl concentrations of 0, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3 and 1.5 M were evaluated (0.3 M was omitted and 1.7 M added for the Q membrane). All conditions were evaluated in duplicate. A gradient for elution in the wells of the well plates was not generated, however, the increasing salt concentration buffers in individual wells enable an estimation of gradient elution. LVs are sensitive to high salt concentration hence samples were diluted immediately following elution with 20 mM Tris/HCl, pH 7.4. Samples eluted in 0.3-0.9 M NaCl were diluted 1:2 and samples eluted in 1.1-1.7 M NaCl were diluted 1:5.

3.2.7 Larger scale runs

Elution conditions determined in the well plate format were tested with 1 mL columns/membranes (CIM Tube Monolithic Column (BIA Separations, Ljubljana, Slovenia) and Sartobind Q, 4 mm bed height (Sartorius Stedim, Epsom, UK)) operated by an ÄKTA Purifier 100 (controlled by Unicorn version 5.11) or an ÄKTA Avant 25 (controlled by Unicorn version 6.1) (GE Healthcare, Buckinghamshire, UK). The sequence of steps was the same as that used for the well plates and the flowrate was 10 mL min^{-1}. Normally for chromatography scaling studies, residence time is kept constant across scales, in this case, however, this was thought to be less critical as mass transfer for membranes and monoliths is convective and not dependent on residence time. In addition, the high linear flow velocity recommended for 1 mL columns is not possible to achieve with the Tecan vacuum manifold.

Feed material was either prepared as for the well plates or clarified cell culture material was loaded directly. In cases where material was first concentrated and then diluted into a loading buffer, 20 mM Tris/HCl, pH 7.4 was used as the loading buffer. This loading buffer with 1.1 M NaCl was the elution buffer for all runs.

A two-level, single block, full factorial design (Table 3.3) was used to study the effect of feed loading volume and volume for elution gradient on LV elution behaviour and protein and DNA removal from the 1 mL Q monolith.

	High (+)	**Low (-)**	**Centre point (CP)**
Feed loading volume (mL)	50	10	30
Volume for elution gradient (CVs)	40	20	30

Table 3.3 Two-level, single block, full factorial design used to study the effect of feed loading volume and volume for elution gradient on physical particle recovery and protein and DNA removal.

3.2.8 Analytical techniques

Infectious titre was determined as described in Section 2.2.4.3, but with two exceptions: (i) filtration of samples with a 0.45 μm syringe filter to remove any microbial contaminants following chromatography operations outside of the laminar air flow cabinet and (ii) sample dilution was based on the expected concentration of vector in the sample, e.g., no dilution of wash samples. Titrations were done immediately following chromatographic experiments as freeze-thaw of purified samples results in significant reduction in titre.

3.2.8.1 Measurement of lentiviral p24 capsid protein concentration

The determination of lentiviral p24 capsid protein concentration was carried out using the Lenti-X p24 Rapid Titer Kit (Clontech Laboratories, Mountain View, USA) according to the manufacturer's instructions. This is a standard "sandwich" ELISA. Each sample was analysed in duplicate and read using a Tecan Infinite M200 plate reader (Tecan, Männedorf, Switzerland). p24 concentration was calculated relative to a standard curve of p24 sample provided in the kit. To ensure samples were in the linear range of the assay, they were diluted according to the estimated p24 concentration.

3.2.9 Total protein determination

Protein concentration was determined using the 96-well plate protocol of the bichinchonic acid (BCA) Protein Assay Kit (Life Technologies, Paisley, UK) according to the manufacturer's instructions.

3.2.10 Quantitation of dsDNA concentration

dsDNA concentration was determined using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Paisley, UR) with a 96-well plate protocol and standard fluorescent wavelengths (excitation 480 nm; emission 520 nm).

3.3 Results and discussion

3.3.1 Microscale monolith chromatography – initial method development

The 96-well monolithic plates used in this study are currently in beta testing and therefore some initial characterisation was carried out to determine flow rates through the resin and to assess well-to-well comparability.

3.3.1.1 Determination of well flow rates

Liquid movement through the chromatographic media in the 96-well monolithic plates can be achieved using either centrifugation or vacuum filtration (BIA Separations instruction manual). In this work, vacuum filtration on an automated liquid handling platform has been used. In order to establish the filtration time required to vacuum a specific volume of liquid through the wells, the flow rates at the chosen operating pressure need to be known. The plate manufacturer recommends operating the vacuum manifold at a pressure difference below -1 barg. The highest pressure achievable with the Te-VacS vacuum system employed in this work is -0.7 barg, and this was chosen to minimise operation time.

Flow-through liquid from a representative selection of wells, shown in Figure 3.1, following pressure application for five different time points was collected and used to calculate the flow rates through the wells. The results, shown in Table 3.4, are homogeneous, with a few central wells (D6, D8, F8, F10) displaying higher flow rates. Sample residence time will be lower in these wells, however, as mass transfer in monolithic columns is based on convection rather than diffusion, this should not lead to a difference in DBC.

It is difficult to achieve a perfect seal between the plate and the vacuum manifold, and it was speculated that the wells along the plate edges would have lower flow rates. However, this was not observed and there appears to be no trend between flow rate and well location. For subsequent work, filtration times were based on a flow rate of 0.25 mL min^{-1} (corresponding to a linear flow velocity of 23 cm h^{-1}), which was based on the wells with the lowest flow rates with an added safety margin, so as to ensure that all the liquid is passed through all the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.37 ± 2.2 × 10^{-2}				0.37 ± 2.7 × 10^{-2}			0.32 ± 2.2 × 10^{-2}				0.34 ± 4.0 × 10^{-2}
B												
C			0.31 ± 3.7 × 10^{-2}							0.37 ± 4.9 × 10^{-2}		
D						0.51 ± 3.1 × 10^{-2}		0.43 ± 2.9 × 10^{-2}				
E	0.36 ± 3.1 × 10^{-2}											0.46 ± 6.6 × 10^{-2}
F			0.34 ± 2.6 × 10^{-2}			0.35 ± 3.4 × 10^{-2}		0.52 ± 2.8 × 10^{-2}		0.40 ± 2.8 × 10^{-2}		
G												
H	0.37 ± 1.7 × 10^{-2}				0.34 ± 3.1 × 10^{-2}			0.36 ± 2.5 × 10^{-2}				0.29 ± 1.3 × 10^{-2}

Figure 3.2 Flow rates (mL min^{-1}) through selected wells on methacrylate monolithic 96-well plate during vacuum filtration (pressure = -0.7 barg). Flow rates were obtained by finding a relationship between flow-through volume and filtration time using linear regression. Errors represent one standard deviation around the mean (*n* = 15).

3.3.1.2 Determination of protein binding capacity

In order for the 96-well monolithic plates to generate meaningful results, all wells must perform similarly with respect to binding capacity. To assess binding capacity for a representative feed, clarified cell culture supernatant containing RDpro pseudotyped LV was loaded onto the Q plate and the total protein concentration of the feed and flow-through, wash and eluate fractions was determined.

Resin binding capacity for total protein, as shown in Figure 3.2 (a), was found to be comparable between all wells, with a relative standard deviation (RSD) of 11%. Likewise, the protein concentration of the wash and eluate fractions had RSDs of 14% and 13%, respectively (Figure 3.2 (b) and (c)). All 96 samples from the plate could not be processed in a single assay run, thus inter-assay variability may be one explanation for the variations seen.

Binding capacities for the wells were calculated using Equation 3.1. The mean binding capacity for the Q resin was 6 mg mL^{-1}, which is low compared to vendor-reported values of ≥ 21 mg bovine serum albumin (BSA) per mL^{-1} of monolithic resin (BIA Separations, 2012). The lower capacity observed in this work could be due to the high ionic strength of the clarified cell culture material loaded.

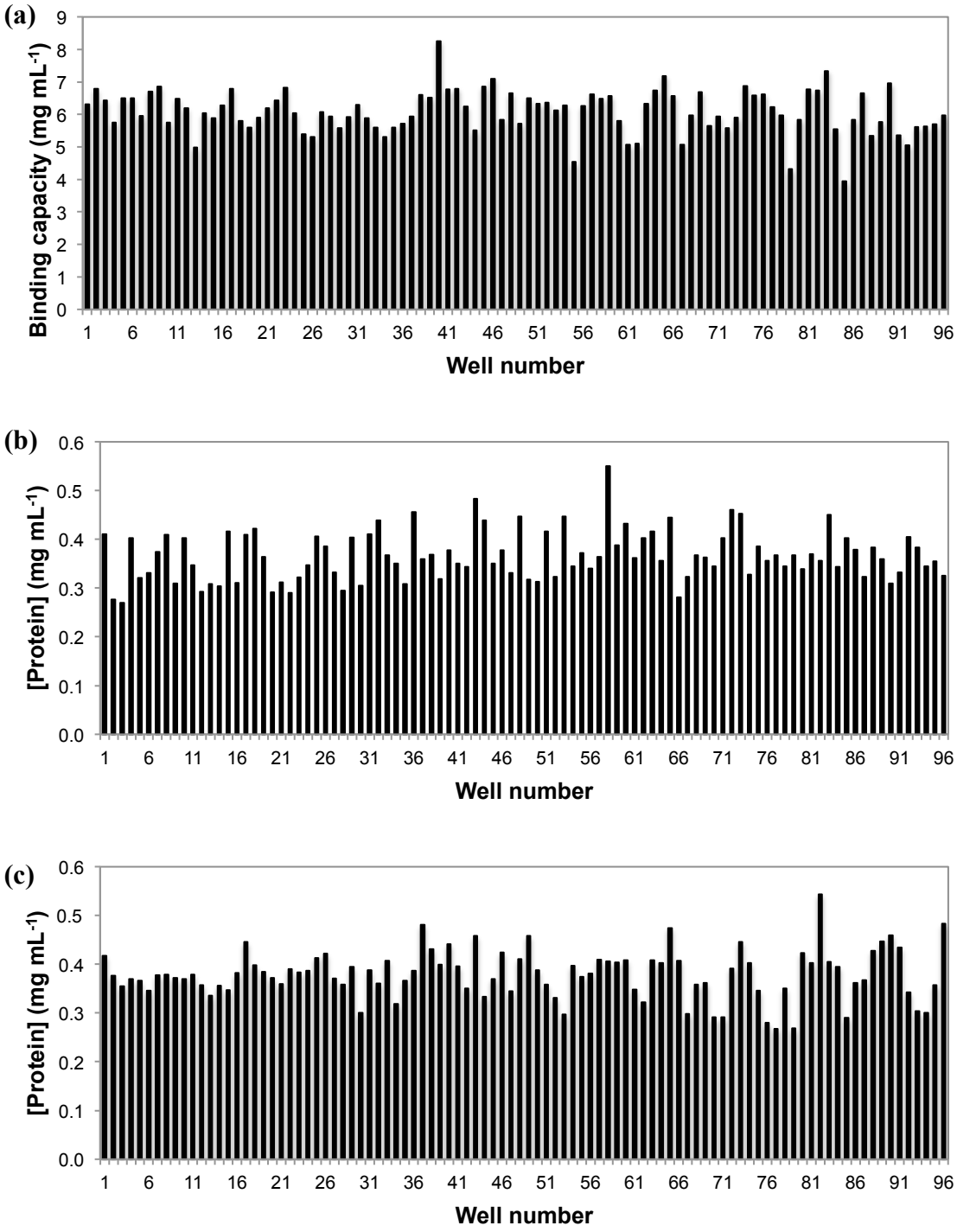


Figure 3.3 **Evaluation of well-to-well comparability on Q methacrylate monolithic 96-well plate. 1 mL of clarified cell culture supernatant from STAR-RDpro cells was loaded per well, each containing 0.2 mL of monolithic resin. In (a) each bar represents the protein binding capacity for an individual well; in (b) each bar represents the protein concentration of the wash buffer for an individual well; in (c) each bar represents the protein concentration of the eluate for an individual well. Protein concentration was determined using a commercial BCA protein assay kit.**

3.3.2 Investigation of loading conditions on Q and DEAE 96-well plates

To establish whether LV DBC could be improved on Q and DEAE monolithic resins, loading buffer species, pH and buffer strength were evaluated. The mobile phase should be selected so as to have minimal interaction with the resin ligands and to have buffering capacity in the pH range of interest. Out of the buffers suitable for AEX, Tris/HCl and HEPES have pK_as at 20°C of 8.30 and 7.55, respectively. This made them suitable for this work where the pH range required was 7-8. Phosphate buffers are theoretically not suitable for AEX as the phosphate ion interacts with the positively charged ligands, nevertheless phosphate buffers are still commonly used successfully for AEX-based separations. Phosphate has a pK_a of 6.86 and is therefore useful in the pH range of interest and was included in the evaluation.

In initial experiments it was found that loading 2 mL of clarified cell culture material per well typically resulted in breakthrough of p24 capsid protein, hence for this study, an equivalent volume of concentrated cell culture material was loaded into each well.

The buffer conditions studied were not seen to be influencing resin binding capacity and hence the results can be visualised with a simple bar chart (Figure 3.3) as opposed to a contour plot. For Tris/HCl and HEPES buffers, LV was found to bind well under all conditions evaluated, whereas a lower DBC was observed for the phosphate buffered samples. For the Q resin, the DBCs observed for phosphate buffer was approximately 2×10^{10} VPs mL^{-1} of resin, whereas DBCs for Tris/HCl and HEPES were approximately 1×10^{11} VPs mL^{-1}. For DEAE, DBCs were slightly lower: 3×10^{10} VPs mL^{-1} for HEPES; 4×10^{10} VPs mL^{-1} for Tris/HCl; and 1×10^{10} VPs mL^{-1} for phosphate. These values are slightly lower than the typical capacities of chromatographic supports reported in the literature of 10^{11}-10^{12} vector particles per mL of resin (Segura et al., 2011).

LVs are large macromolecules with multiple sites available for binding, hence they exhibit increased binding to anion exchangers compared to contaminating proteins. Loading can therefore be carried out at high ionic strength, where selectivity is maximised and therefore DBC increased. In the interest of reducing the number of processing steps and time, for LVs, it seems sensible to omit buffer exchange prior to loading onto the chromatographic capture step.

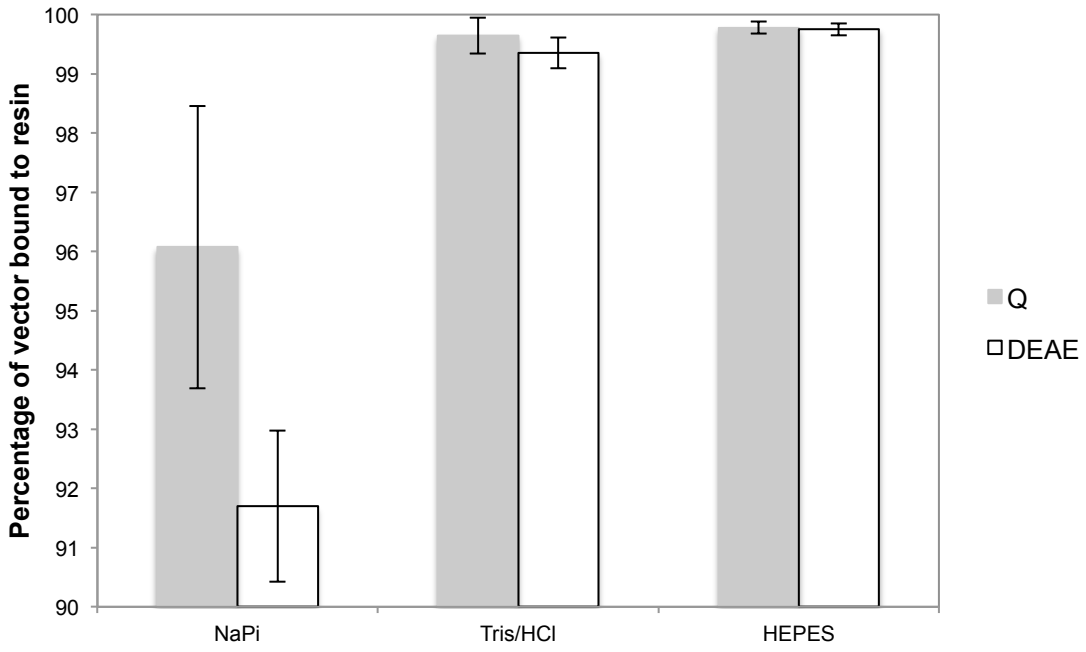


Figure 3.4 A comparison of the effect of loading buffer composition on the binding of LV to Q and DEAE monolithic resins. Three different buffer species (sodium phosphate (NaPi), Tris/HCl and HEPES) were evaluated; buffer concentration was varied from 20 to 60 mM; and pH from 7 to 8.5. Clarified vector supernatant was concentrated 10-fold and immediately diluted 10-fold into the different loading buffers. The percentage of bound vector was found by analysing the quantity of p24 HIV capsid protein by ELISA in the feed and the flow-through. Error bars represent one standard deviation around the mean (*n* = 16).

3.3.3 Investigation of elution conditions

Following poor performance for sodium phosphate buffer and similar performance for Tris/HCl and HEPES during binding studies, it was decided to carry out elution studies with Tris/HCl as this is a more cost effective buffer system. Elution from AEX resins can be carried out by increasing the ionic strength or altering the buffer pH. pH elution is unlikely to be suitable for LVs as they have a low pI and significantly changing pH will lead to vector inactivation. Elution by increased salt concentration is also problematic as vector stability is optimal at physiological ionic strength (Segura et al., 2005). Dilution immediately following salt elution alleviates vector inactivation as shown by Bandeira et al. (2012) for a VSV-G-pseudotyped LV. Figure 3.4 shows that recovery is reduced by 10% for the vector used in these studies unless the eluate is diluted. The recoveries seen in Figure 3.4 are low and were in this case possibly due to the purified and diluted eluates being frozen prior to titration. The disadvantage of

dilution of the eluate is that it reduces the benefit of concentration following the chromatography step. However, the minimal dilution that retains infectivity should be found as opposed to just evaluating one dilution factor.

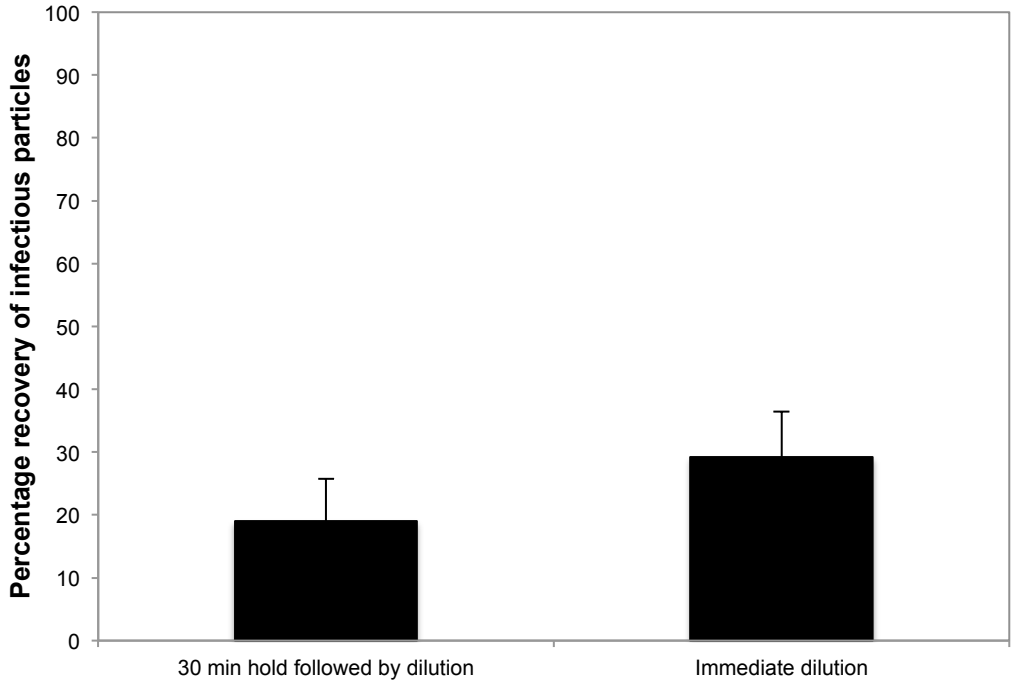


Figure 3.5 The effect of eluate dilution immediately following elution compared to eluate left for 30 min before dilution on the yield of infectious LV particles. Clarified vector supernatant was loaded into wells of a microtitre plate containing Q monolith resin and eluted with 20 mM Tris/HCl, pH 7.4, with 1.1 M NaCl. One set of samples was immediately diluted 1:5 with elution buffer without NaCl and the second set left untreated. Both were stored at 4°C for 30 min before diluting the second sample set, followed by freezing feed and eluates at -80°C prior to titration on HEK 293FT cells. Error bars represent one standard deviation about the mean (*n* = 3).

Figure 3.5 shows the results of elution by increased salt concentration from monolithic resin with Q or DEAE ligands and membrane with Q ligands. The purpose of this experiment was to conduct an initial screening of the various types of resin available in a 96-well format. The vector elutes most readily from the DEAE resin, however, the recovery of infectious particles is the lowest. The Q membrane and Q monolith require higher salt concentration for vector elution. From the Q monolith the recovered vector has a high infectivity (ratio of infectious to physical particles), which means vector product of high quality. A high level of the vector recovered from the Q membrane is infectious, however, the yield is only approximately 50% from this resin type. It is worth noting that for some conditions (e.g., Figure 3.5 (a) 1.1 M NaCl) recovery of infectious particles appears to be higher than the recovery of total particles. These instances are always within the standard deviations of the results and in addition this highlights an important limitation of the cell-based titration method: the inherent variability of the method means that this type of assay is typically qualified with an approximately 30% error margin.

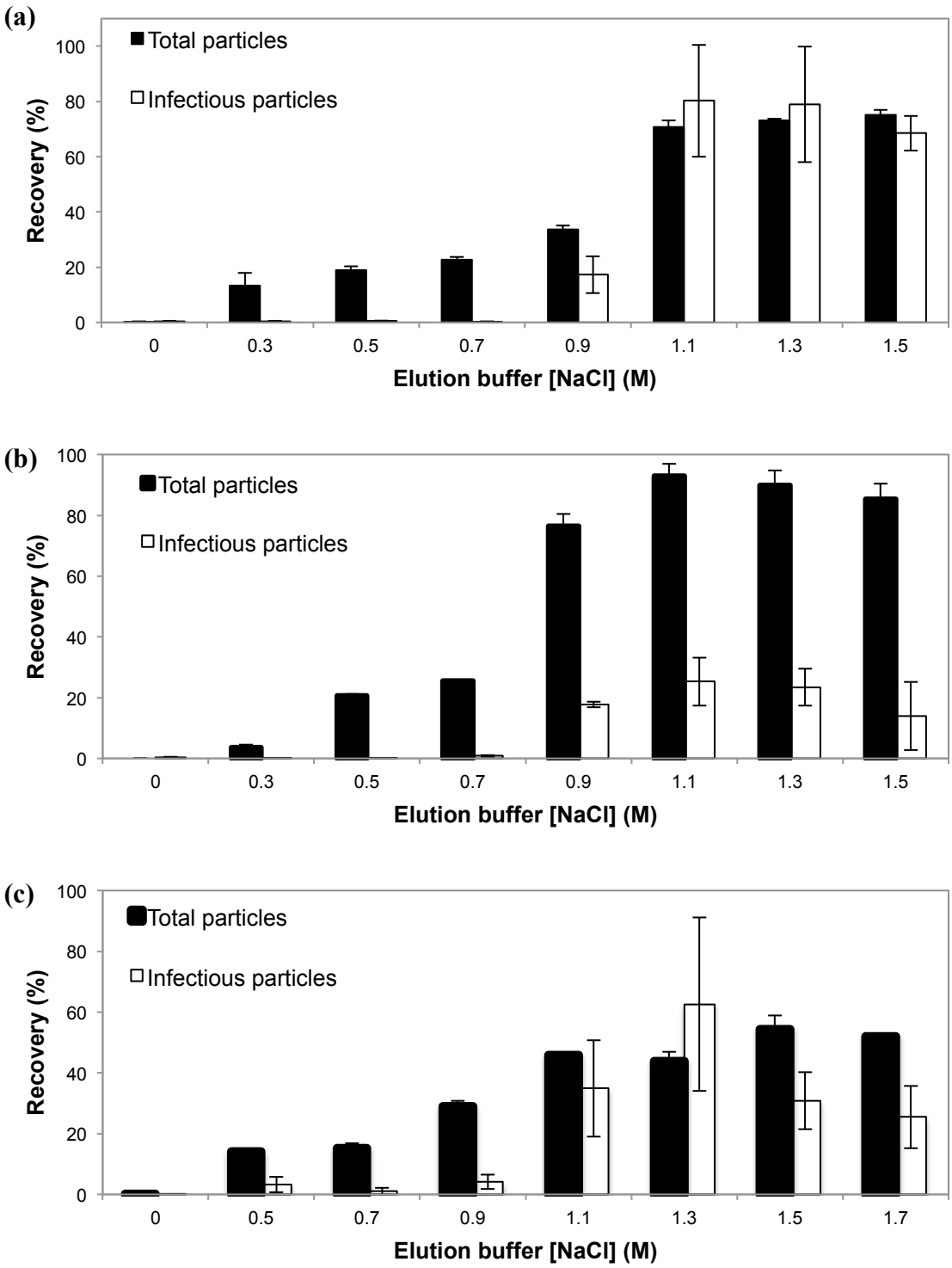


Figure 3.6 A comparison of the effect of elution buffer [NaCl] on the recovery of total LV particles and infectious LV particles. In (a) the recovery from wells containing monolithic resin with Q ligands; in (b) the recovery from wells containing monolithic resin with DEAE ligands; and in (c) the recovery from wells containing membrane with Q ligands. The total particle concentration was determined by p24 ELISA and the infectious particle concentration by titration on HEK 293FT cells and read-out by flow cytometry. Values shown are the mean of two experiments and error bars show the actual data points.

Figure 3.6 shows the percentage of protein and dsDNA removed from sample loading to elution for the different elution conditions. In Figure 3.6 (a), protein removal can be seen to remain almost constant once some salt is added to the elution buffer. This is expected as protein elutes more readily than LV and DNA. Total protein measurement will include protein components of the LV, hence a HEK 293 host cell protein ELISA would be a better measurement of impurity removal. Protein removal does not decrease as LV elutes (1.1 M NaCl for Q monolith; 0.9 M NaCl for DEAE monolith, 1.1 M NaCl for Q membrane) which could be interpreted as if the protein contribution from LV particles is negligible compared to the total protein bound to the resin.

DNA is tightly bound to the resins as can be seen in Figure 3.6 (b) where removal is almost complete until the elution buffer contains 0.7 M NaCl and DNA starts to elute. The Q membrane and DEAE monolith follow a similar trend where removal drops to a constant level of approximately 20% once the elution buffer contains 1.1 M NaCl or more. These 20% most likely represent the dsDNA collected in the flow-through during loading. The Q monolith follows an unexpected trend where DNA removed reaches a minimum for 0.9 M NaCl and then increases again and appears to plateau at 80% removed. NaCl is known to interfere with the PicoGreen assay used for DNA quantitation, however, samples were diluted to reduce the NaCl concentration and samples from all three resins were treated the same.

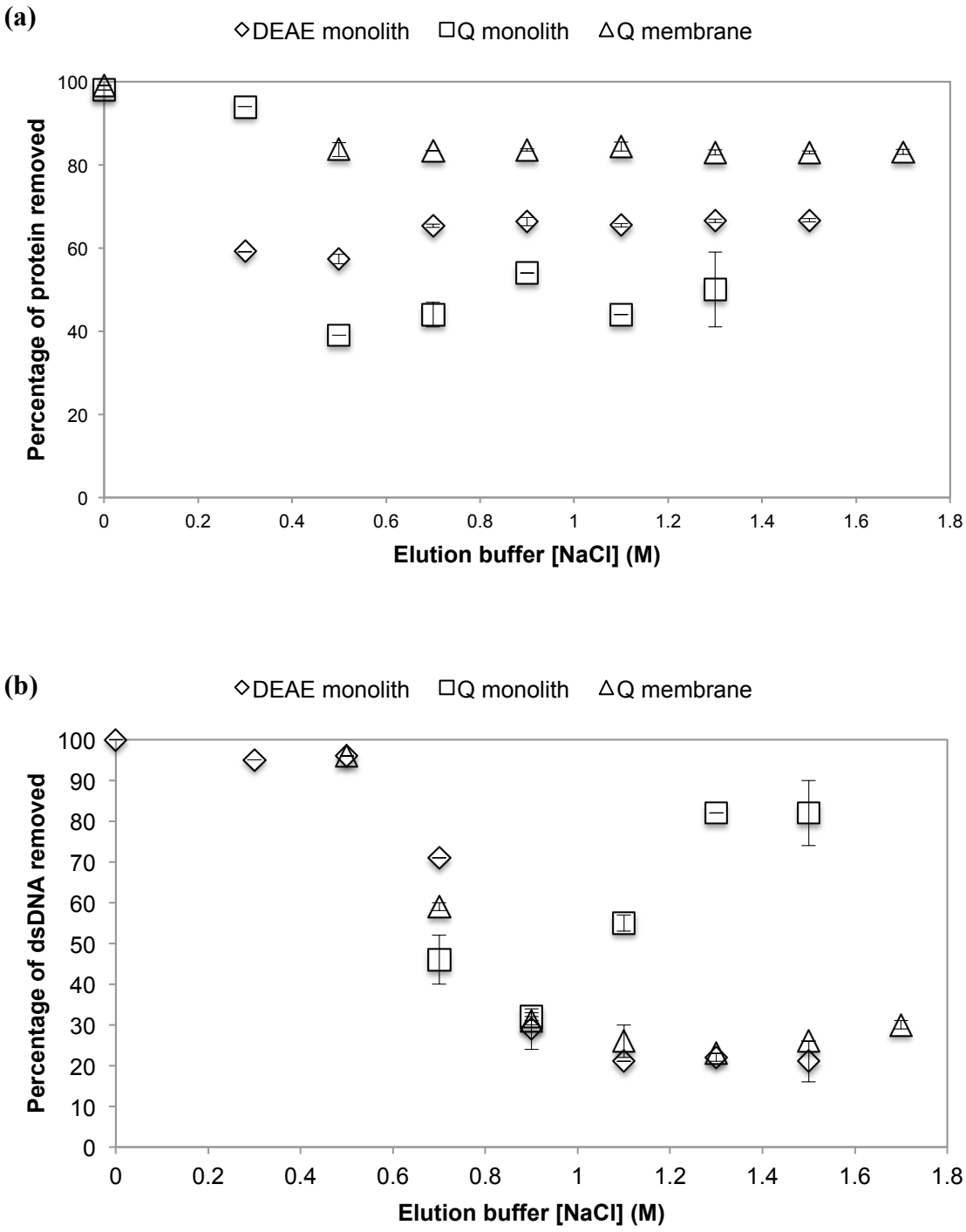


Figure 3.7 **A comparison of the effect of elution buffer NaCl concentration on the removal of (a) total protein and (b) dsDNA. Protein and dsDNA concentration were determined using commercial assay kits. Values shown are the mean of two experiments and error bars show the actual data points.**

3.3.4 Verification of 96-well plate results on laboratory scale columns

In 96-well plate-based experiments, infectious vector yield was found to be highest with the Q monolith, as described in Section 3.3.3, hence a 1 mL version of this resin was chosen for lab-scale experiments along with a 1 mL Q membrane capsule.

Initial runs were carried out where feed material was prepared in the same manner as for elution experiments in plate format (Section 3.3.3) and the load to resin volume ratio was kept the same (Figure 3.7 (a)), or increased (Figure 3.7 (b)-(d)), compared to the well plate format. The chromatograms are shown in Figure 3.7. The majority of bound material can be seen to have eluted at approximately 40% of elution buffer concentration during gradient elution. This is contradictory to the results obtained in plate format as at 0.5 M NaCl, only a small recovery of vector was obtained. To evaluate if this result was related to the elution gradient or feed volume, a two-level, single block, full factorial experimental design was carried out using the Q monolith, as described in Table 3.3.

Figure 3.7 (d) is an example chromatogram from the Q membrane capsule. The elution resolution is worse compared to the monolith, despite this run being carried out with a 40 CV gradient compared to the 20 CVs used for elution from the monolith. Even during loading the absorbance reading fluctuates, most likely due to mixing in the void volume of the capsule. Due to the poor resolution, the remainder of the work was carried out with the Q monolith only. It is worth noting that elution from the Q membrane started at a similar elution buffer salt concentration to the Q monolith, thus this result was not simply an anomaly for the monolithic stationary phase.

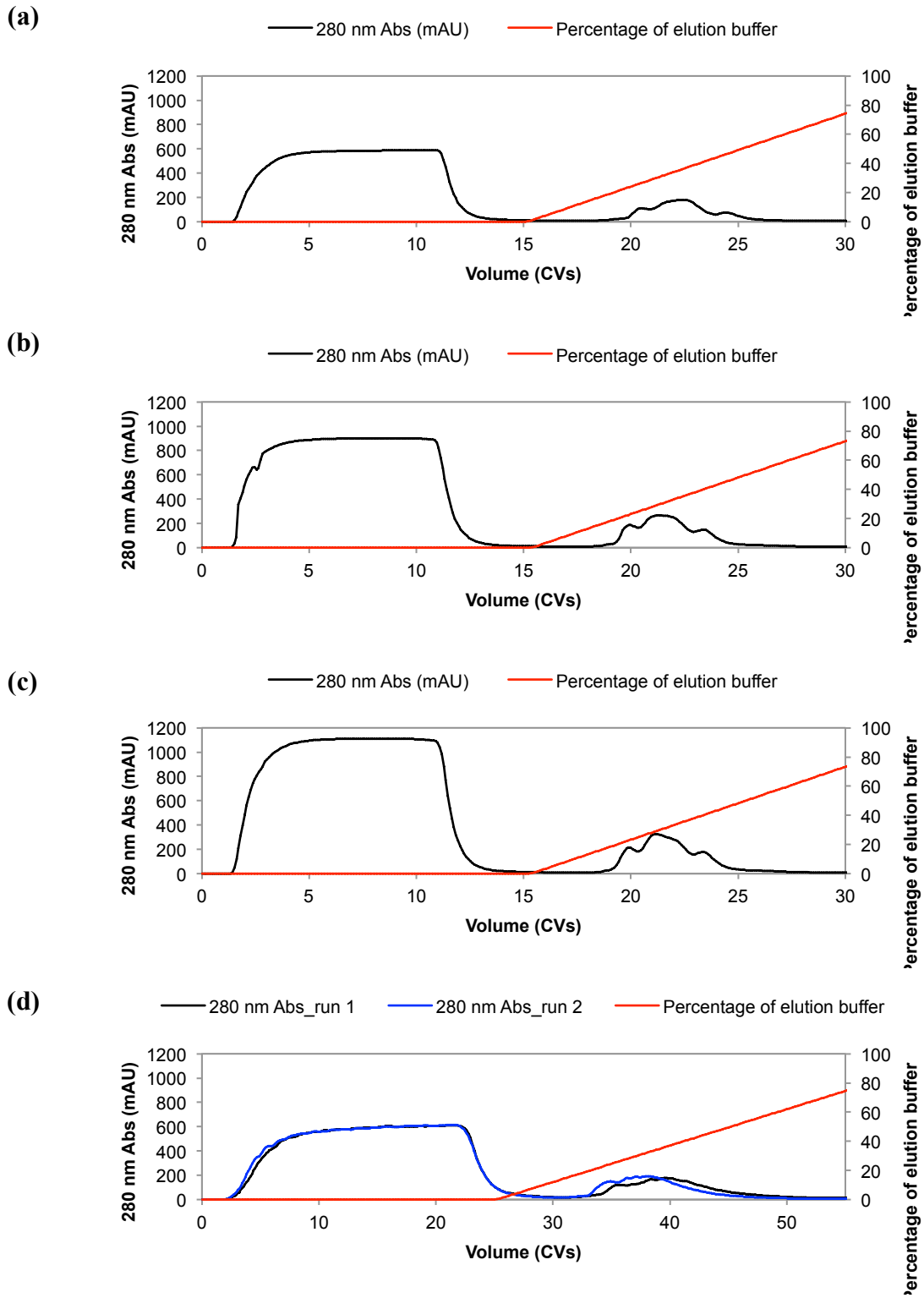


Figure 3.8 Chromatograms for varying concentrations of feed loaded onto a 1 mL Q monolith column, (a)-(c), or 1 mL Q membrane, (d). (a) 10 mL clarified cell culture material loaded onto monolith; (b) 10 mL 2 x concentrated cell culture material loaded onto monolith; (c) 10 mL 3 x concentrated cell culture material loaded onto monolith; (d) 20 mL clarified cell culture material loaded onto Q membrane. Elution was carried out with a linear gradient of 0-100% elution buffer (20 mM Tris-HCl, pH 7.4 with 1.1 M NaCl).

3.3.4.1 Two-level, full factorial design study on 1 mL Q monolith investigating the effect of feed load volume and elution gradient steepness on lentiviral vector elution behaviour

Two factors were considered in this study: feed volume and number of column volumes (CVs) for elution. Feed volume was varied between 10 and 50 mL, with a centre point (CP) at 30 mL, whereas gradient was varied between 20 and 40 CVs, with a CP at 30 CVs. Two CP runs were carried out and in Figure 3.8 and 3.9 (e) these can be seen to be highly reproducible.

Figure 3.8 (chromatograms with LV concentrations) and 3.9 (chromatograms with dsDNA and total protein concentrations) show that three elution peaks are distinguishable in all runs: the first peak corresponds to elution of contaminating proteins, the second peak corresponds to elution of LV and the final peak corresponds to elution of DNA.

When collecting the LV peak, all runs removed 98% or more of total protein. DNA removal, however, was dependent on elution gradient, with the increased resolution offered by a shallower gradient resulting in more efficient removal. For runs with a 40 CV elution gradient, more than 95% of DNA was removed, whereas for runs with a 20 CV elution gradient, removal was more than 60%. If the anion exchange capture step is followed by endonuclease treatment and UF or SEC, DNA removal could be considered unimportant and an elution strategy that maximises LV recovery, without considering DNA removal, could be adopted. Protein is primarily removed during loading and is less dependent on elution resolution. Despite the vast majority of protein being removed during AEX capture, it is worth pointing out that some difficult-to-remove proteins may be undesirable in the final vector formulation as McNally et al. (2014) showed that for a γ-retroviral vector, serum proteins remained after purification by a Q membrane.

p24 concentration in a fraction collected towards the end of the loading phase is low for all loading volumes, suggesting that the capacity of the resin is higher than the capacity determined in well plates (based on those results, p24 breakthrough should occur after loading approximately 10 mL of LV-containing feed). For the experiment in well plates, breakthrough of p24 was used as an indicator of LV capacity. This may have been a mistake as what was breaking through was most likely p24 protein not associated with viral particles, and the capacity for viral particles was in fact higher than

suggested by the well plate data. This highlights an important limitation of the use of p24 ELISAs to detect LV: there is no distinction between p24 associated with particles and p24 not packaged into viral particles. However, there is now a marketed p24 ELISA kit available ("Lentivirus-Associated p24 ELISA Kit" from Cell Biolabs) that claims to be able to distinguish between free p24 and capsid p24. Not loading to true breakthrough capacity is a likely explanation for the slightly low DBCs obtained in Section 3.3.2.

Physical particle recovery was found to be dependent on load volume, but not on elution gradient length. Only 9% of LV particles were recovered when the load was 50 mL, despite no significant breakthrough being detected. The recoveries for 10 and 30 mL loads were all approximately 20%. These recoveries are significantly lower than in well plates where the yield was more than 70% for NaCl elution concentrations above 1 M. These results cannot be explained by shear damage in chromatography system pumps and tubing as this is physical particle, rather than infectious particle, recovery. It is possible that particles get trapped in monolith pore constrictions, as discovered by Trilisky (2009) for an adenoviral vector.

For all runs, LV eluted much before the elution buffer had the ionic strength required for elution from monolith and membrane in well plate format. Figure 3.10 shows that the start of LV elution is dependent on the steepness of the gradient, but not on the feed loading volume. LV starts to elute earlier when the gradient is shallower (higher CVs for elution). This suggests that elution could be dependent not only on the ionic strength of the buffer, but also on the contact time with elution buffer. In the well plates elution was carried out over 8 min whereas LV had eluted from the 1 mL column within 2 min. The linear flow velocity in wells was 23 cm h^{-1} compared to 390 cm h^{-1} (this is an average velocity as the 1 mL monolith is tubular and flow is radial) on the 1 mL monolith column. It is possible that shear effects from the higher flow velocity aids in disrupting binding between vector and matrix.

Although no published reports of purification of an RDpro pseudotyped LV by AEX exist, LVs with other pseudotypes (VSV-G and measles virus glycoproteins) elute at NaCl concentrations around 0.5 M, e.g, 0.65 M (Bandeira et al., 2012) and 0.2-0.4 M (Marino et al., 2015). The high-throughput plate formats therefore may need further development to be useful for screening of LV elution conditions.

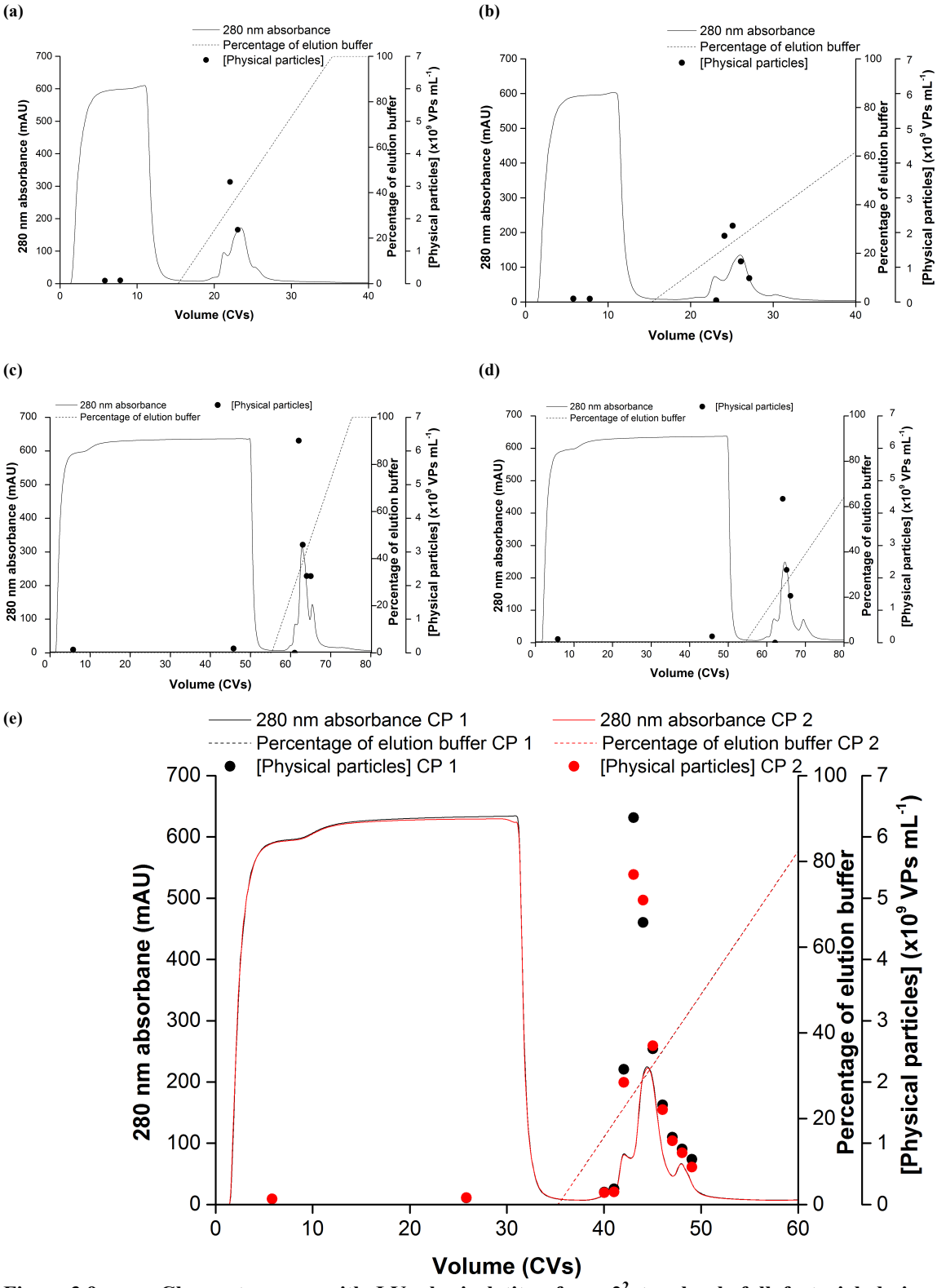


Figure 3.9 **Chromatograms with LV physical titre from 2^2 two-level, full factorial design investigating the effect of feed loading volume and elution gradient steepness (CVs for elution) on elution behaviour of a LV. In (a) 10 mL load (-), 20 CV elution (-); (b) 10 mL load (-), 40 CV elution (+); (c) 50 mL load (+), 20 CV elution (-); (d) 50 mL load (+), 40 CV elution (+); (e) 30 mL load, 30 CV elution (CP). The physical particle concentration was determined by p24 ELISA.**

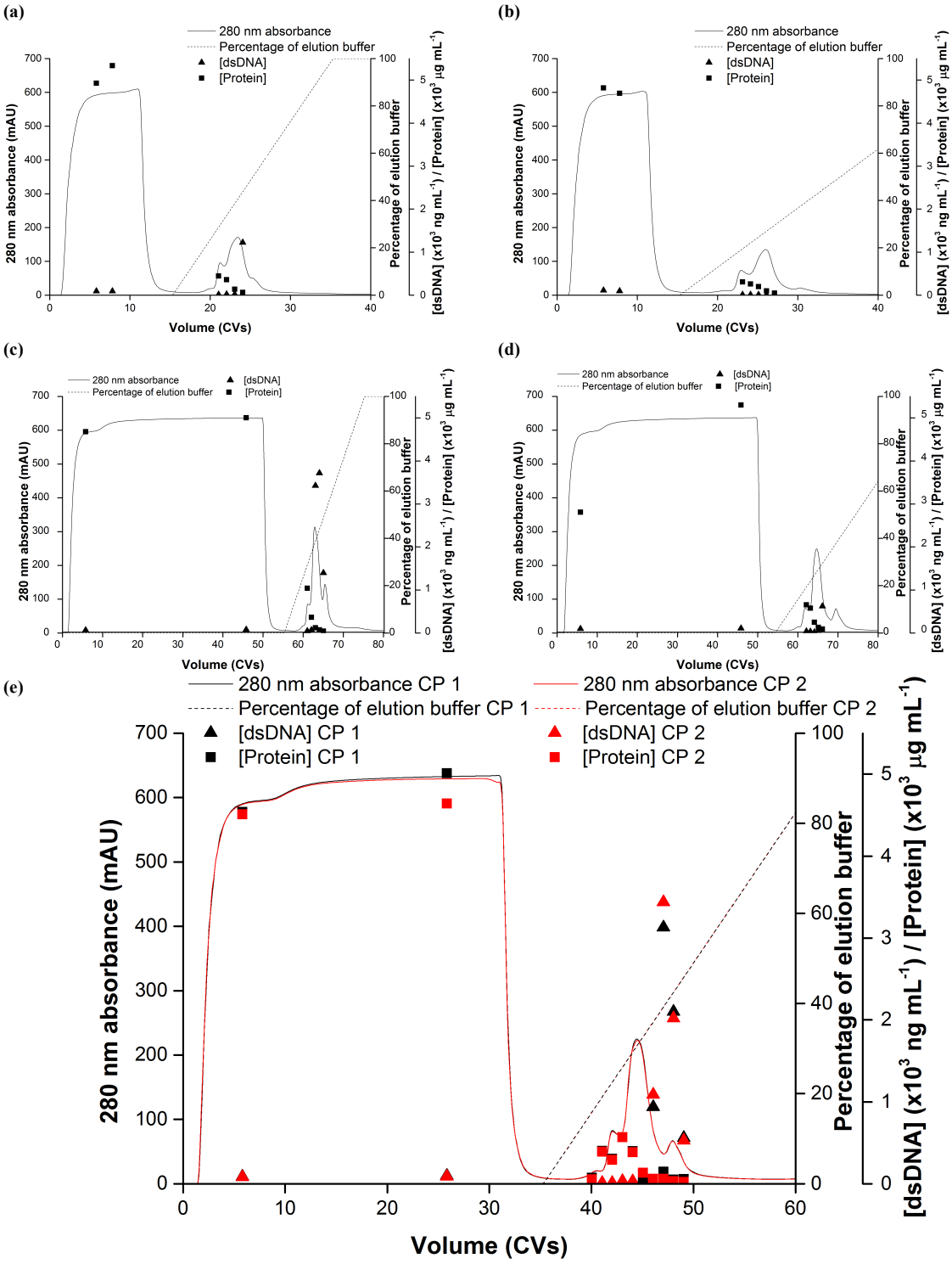


Figure 3.10 **Chromatograms with dsDNA and total protein concentrations from 2^2 two-level, full factorial design investigating the effect of feed loading volume and elution gradient steepness (CVs for elution) on elution behaviour of a LV. In (a) 10 mL load (-), 20 CV elution (-); (b) 10 mL load (-), 40 CV elution (+); (c) 50 mL load (+), 20 CV elution (-); (d) 50 mL load (+), 40 CV elution (+); (e) 30 mL load, 30 CV elution (CP). The dsDNA and total protein concentrations were determined with commercially available kits.**

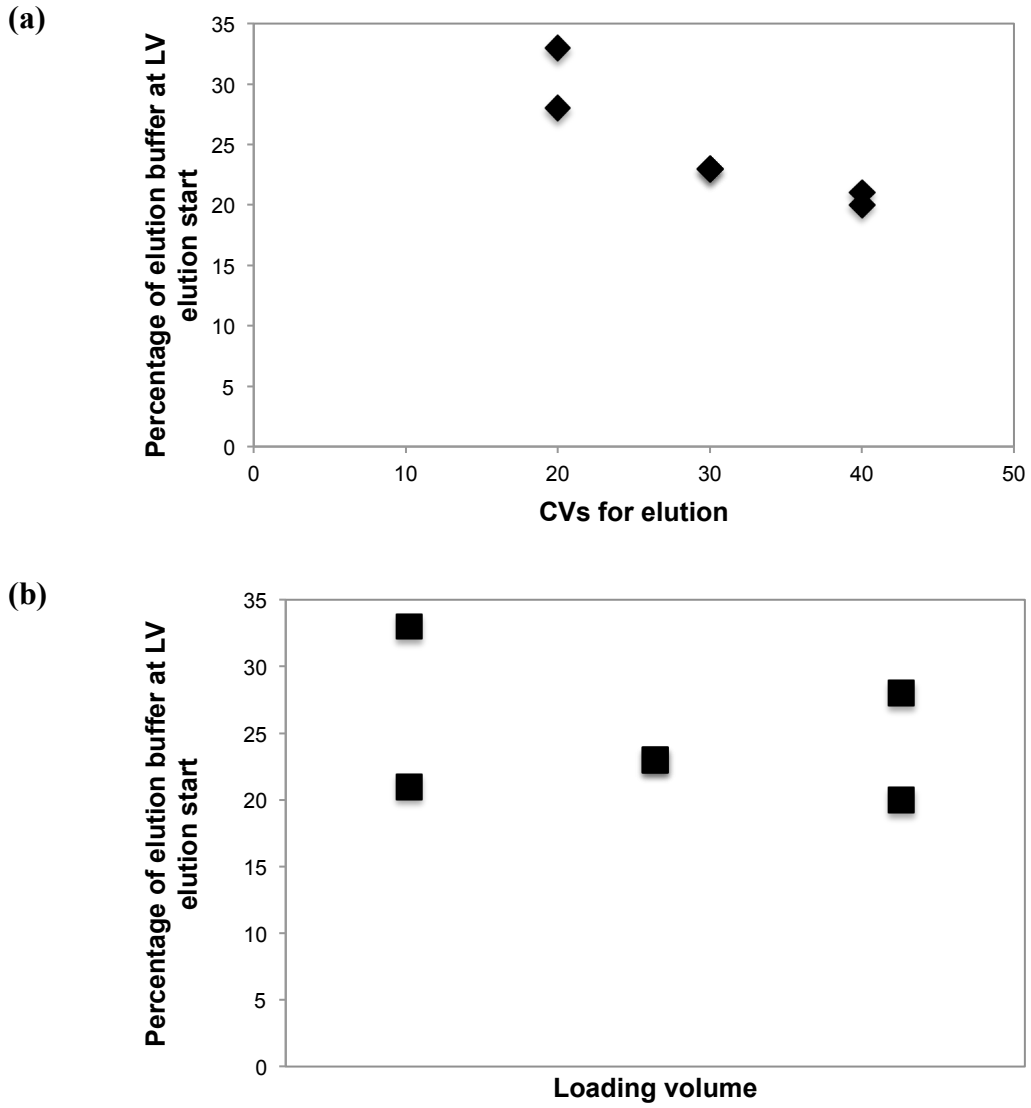


Figure 3.11 **Scatter plots illustrating the effect of (a) gradient steepness and (b) feed loading volume on the ionic strength of elution buffer required to elute LV from a 1 mL Q monolith resin. A two-level full factorial design was carried out as described in Table 3.3. The elution buffer was 20 mM Tris/HCl, pH 7.4 with 1.1 M NaCl.**

3.4 Conclusion

High-throughput 96-well monolith plates were found to have reproducible protein binding capacities and were deemed suitable for initial development of an AEX-based capture step for a LV. Binding studies carried out in the plate format showed that vector binding was efficient under all conditions tested, except when using a phosphate buffer as the binding buffer. This is not unexpected as the phosphate ion will interact with the positively charged AEX resin. Elution studies in the plate format established that a salt concentration around 1 M was required to elute LV from both a monolithic stationary phase (Q and DEAE ligands) and a membrane stationary phase (Q ligand). Immediate dilution of the eluate with buffer without salt was found to be beneficial for retention of infectious titre.

Studies with a 1 mL Q monolith and 1 mL Q membrane operated by an ÄKTA chromatography system found that much lower salt concentrations than those required in well plates were sufficient to elute LV particles. The difference may be related to the low flow velocity in plates compared to 1 mL capsules. Recoveries of physical particles were several times lower in 1 mL capsules than in the plate format. These results suggest that AEX elution conditions for LVs are preferably evaluated on a system as similar as possible to that to be used for manufacturing.

4. FORMULATION OF AN RDPRO PSEUDOTYPED HIV-1-DERIVED LENTIVIRAL VECTOR

4.1 Introduction

In this chapter, thermostable formulations of LV are explored. It is not expected that LV formulations need to be thermostable to make products currently in development commercially viable, although it would be practical and beneficial from a COGs perspective if the requirement for storage in the frozen state could be avoided. In this study, an LV was used as an example of a difficult-to-stabilise virus. The applications where thermostable formulations of viruses are urgently needed are for prophylactic vaccines (Brandau et al., 2003). Infectious diseases account for more than 17 million deaths per year and particularly burden the developing world, where one in two deaths are due to communicable diseases (World Health Organisation, 1996). Vaccines are a powerful tool in the fight of infectious diseases and it has been estimated that they prevent more than three million deaths per year (Ulmer et al., 2006). However, vaccines are still underutilised in the poorest countries and one reason for this is the need for a 2-8°C refrigerated "cold chain" – the system of refrigerated containers used to maintain protective temperatures – when transporting vaccines (Brandau et al., 2003). In developing countries, the cost of the cold chain can account for up to 80% of the cost of vaccination programs (Kols and Sherris, 2000). The cold chain is needed as vaccines are typically temperature sensitive and maintenance of a specified temperature range during handling is required to retain bioactivity (Braun et al., 2009). Temperature variations lead to vaccine wastage, immunisation recall and subtherapeutic doses if damaged products are not identified. Wastage has been described to be as high as 50% in developing countries, with a large proportion of this attributable to temperature instability (Lloyd, 1999).

The most common approach to vaccine stabilisation has been through formulation (Brandau et al., 2003). Traditional biopharmaceuticals and viral vaccines have been stabilised with excipients such as amino acids and sugars. The stabilising effects of these compounds are thought to be primarily related to preferential hydration effects that stabilise the native state over the unfolded state. Proteins, e.g., albumin, have also commonly been used for viral vaccine stabilisation. The mode of action may be related to nonspecific interactions with container surfaces, thereby preventing damage and/or

product loss through container interaction. Silk proteins have been found to stabilise viruses and for these proteins, the mode of action is thought to be due to the silk protein structure minimising water content and reducing protein chain mobility (Zhang et al., 2012).

The formulation of LVs is currently, in the scientific literature, an almost unexplored area. This may be due to the presence of many other challenges further upstream in the processes and the lack of commercialised treatments based on LVs. In terms of the product concentration, the challenge for LVs is not as significant as for e.g., mAbs, where a considerable dose needs to be delivered in a volume manageable for injection (Daugherty and Mrsny, 2006). The MOI needed for transduction is such that the vectors need to be formulated at a concentration one or two orders of magnitude higher than the cell culture titre, i.e., at a concentration of 10^8-10^9 TU mL^{-1} (Aiuti et al., 2013; Biffi et al., 2013). If therapeutic applications emerge where a high MOI is needed for transduction, a larger volume of vector can simply be added.

LVs for clinical protocols have been formulated as liquids and stored at ≤-65°C and transported in the frozen state. Compared to parenteral products, there is more flexibility with respect to the formulation buffer. The vast majority of LV treatments are carried out *ex vivo* and the transduced cells are washed before reinfusion into the patient, hence it is possible and convenient to formulate the vector into the medium used for transduction. Commonly used media are Lonza's X-VIVO™ and CellGenix's CellGro® (Aiuti et al., 2013; Biffi et al., 2013; O. W. Merten et al., 2011). Both contain proteins and sugars and are therefore likely to offer some cryoprotection. In a report from a CMO, LV was formulated in PBS with 0.04 g L^{-1} of lactose (Ausubel et al., 2012). A generic formulation buffer is likely to be practical for a CMO whose customers may use different transduction media. Carmo et al. (2008) found that MLV vectors could be stabilised in liquid formulations containing recombinant human serum albumin (HSA), whereas for a LV, HSA along with a lipoprotein solution derived from bovine serum was required to improve stability compared to a Tris-based buffer. This result is interesting as it highlights that LVs are more difficult to stabilise than γ-retroviral vectors, however, animal-derived components are undesirable from a regulatory perspective and other methods of LV stabilisation would have to be used for commercialised treatments.

Freeze-drying of LV has not yet been reported, however, there have been reports of freeze-drying of MLV vectors with good recoveries following reconstitution (Cruz et

al., 2006; Kotani et al., 1994). In this work, the approach described by Stewart et al. (2014) – inspired by the stabilising compounds found in plant seeds – is utilised. This involves blends of sucrose and raffinose in varying ratios, combined with a derivative of glycine.

Chapter aims and objectives:

- to determine the impact of freeze/thaw cycling on infectious vector titre;
- to compare lyophilisation formulations based on their capacity to retain infectious LV titre following storage for different periods of time at a range of temperatures; and
- to compare liquid formulations based on their capacity to retain infectious LV titre following heat challenge for up to one week at 40°C.

4.2 Materials and methods

4.2.1 Lentiviral vector-containing material

LV was generated as described in Section 3.2.1, followed by concentration as described in Section 2.2.3. To generate highly concentrated vector stock, concentrated material was pooled and further concentrated.

4.2.2 Freeze-drying screening and optimisation studies

Formulations (Table 4.1 and Table 4.2) were made up in PBS and sterilised by filtration through a 0.22 μm syringe filter. Triplicate vials were set up with 50 μL of LV material added to 250 μL of formulation or PBS control in 2 mL glass vials (Schott, Mainz, Germany). Vials for lyophilisation had rubber bungs (Schott, Mainz, Germany) partially inserted and were loaded into an AdVantage freeze-dryer (VirTis, Gardiner, USA) and lyophilised. Following lyophilisation, samples were capped under vacuum, removed, crimped and placed at 37°C for one week of thermochallenge.

Formulation	Composition
A	0.8 M methylsulfonylmethane (MSM), 0.7 M dimethylglycine (DMG), 0.5 M sucrose
B	1 M DMG, 0.5 M sucrose, 0.15 M raffinose
C	1.14 M trimethylglycine (TMG), 1 M sucrose, 0.142 M raffinose
D	0.5 M sucrose
E	1 M sucrose, 0.142 raffinose
F	PBS only

Table 4.2 Formulations evaluated in freeze-drying screening study.

Formulation	Composition
C1	1.14 M TMG, 1 M sucrose, 0.135 M raffinose
C2	1.14 M TMG, 0.5 M sucrose, 0.135 M raffinose
C3	0.7 M TMG, 1 M sucrose, 0.135 M raffinose
C4	0.7 M TMG, 0.5 M sucrose, 0.135 M raffinose
C5	0.5 M sucrose, 0.135 M raffinose
C6	PBS only

Table 4.3 Formulations evaluated in freeze-drying optimisation study.

4.2.3 Freeze-drying long-term study

The best formulation from the screening studies was taken into a longer-term study. Triplicate vials were set up for time points at one week, two weeks, four weeks, eight weeks and 12 weeks at temperatures of 4°C, 25°C and 37°C.

Formulation	Composition
C4	0.7 M TMG, 0.5 M sucrose, 0.135 M raffinose
C6	PBS only

Table 4.4 Formulations evaluated in long-term freeze-drying study.

4.2.4 Liquid formulation study

Formulations were sterilised by filtration through a 0.22 μm syringe filter. 50 μL of LV material was added to 250 μL of formulation or PBS control in 2 mL cryovials (Thermo Fisher Scientific, Waltham, USA). Triplicate vials were set up for time points at 1 day, 4 days and 7 days and incubated at 40°C.

Formulation	Composition
1	0.4 M DMG, 0.5 M sucrose, 0.272 M raffinose
2	0.87 M TMG, 0.87 M DMG, 0.5 M sucrose
3	0.7 M TMG, 0.5 M sucrose, 0.135 M raffinose
4	0.5 M sucrose, 0.272 M raffinose (control)
5	0.5 M sucrose (control)
6	TE/PBS buffer only (control)

Table 4.5 Formulations evaluated in liquid formulation study.

4.2.5 Lentiviral vector titration

Infectious LV titre was determined as described in Section 2.2.4.3, with the exception of dilution volume. Prior to titration, lyophilised samples were reconstituted in 300 μL of DMEM-based medium (as described in Section 2.2.1.2) and diluted as described in Table 4.1. Liquid formulations were diluted 1:20 with PBS prior to titration.

4.3 Results and discussion

4.3.1 The effect of freeze/thaw cycling on infectious vector titre

To determine whether freshly harvested and frozen vector could be used interchangeably, the effect of freeze/thaw cycling on vector infectious titre was determined. Vector stability during freeze/thaw cycles was studied by titration on HEK 293FT cells after cycling between -80°C and 37°C. The results in Figure 4.1 show that following the first cycle, the titre was reduced by 25% and after the second cycle, 58% of the initial titre has been lost. These results show a more significant decrease than that observed by Strang et al. (2004) for the same vector. In their study, less than 20% was lost after the first cycle and after the second cycle less than 40% of the initial titre had been lost. The results do, however, show a similar trend and the variation, if statistically significant, could possibly be explained by the difference in the cell line used for titration and the inherent variation of the LV titration method (≈30%). Lee et al. (1996) found that the titre of a γ-retroviral vector was similarly impacted by freeze/thaw as the LV in our study, as following one freeze/thaw cycle titre was reduced by 30-50%.

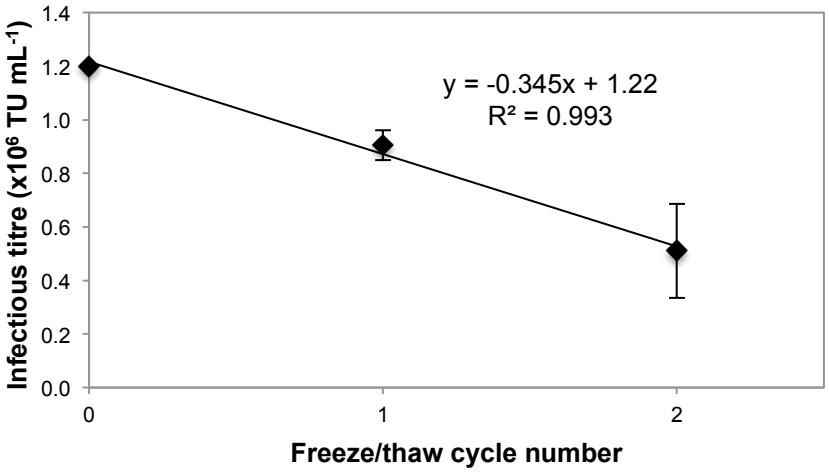


Figure 4.1 The effect of freezing at -80°C followed by thawing on infectious titre. Clarified vector material was concentrated 10-fold and one set of samples was immediately titrated with another two sets of samples frozen at -80°C. Following 24 h, samples were defrosted and one sample set titrated and one sample set once again frozen. The final sample set was titrated 24 h later. Error bars represent one standard deviation about the mean (*n* = 3).

4.3.2 Study of freeze-dried formulations

4.3.2.1 Development of vector titration method

The first time vector was formulated in lyophilisation buffer and titrated on HEK 293FT cells to determine the pre-lyophilisation titre, a wide range of titres was obtained. This was despite the same amount of vector being added to all formulations. The titre was found to correlate with the number of events detected during flow cytometry (Figure 4.2). The flow cytometry events represent particles detected and should primarily consist of single cells, although some cell debris and cell clumps will inevitably be present. The low number of events for formulations A-E indicates that these formulations caused cell death. Formulation F consisted of PBS and an accurate titre was obtained for this formulation. The negative control had the same number of events as formulation F, and as the negative control was PBS without vector added, this was the expected result. The low number of events for formulations A-E prevented an accurate value of the number of eGFP-positive cells to be determined. The number of events detected increased as formulation ionic strength decreased, indicating that osmotic shock may have been the reason for cell death. To enable titration, a range of dilutions and two diluents (cell culture medium and 18.2 MΩ water) were investigated

for each formulation and Table 4.1 shows the dilution factors and diluent that gave comparable titres for formulations A-E to a PBS control (formulation F).

Formulation	Dilution factor / diluent	Percentage of PBS control titre
A	1:18 / culture medium	94
B	1:20 / culture medium	99
C	1:24 / 18.2 MΩ water	111
D	1:14 / culture medium	106
E	1:24 / culture medium	99

Table 4.5 The dilution factor and diluent necessary to obtain a titre comparable to the PBS control for freeze-drying formulations A-E.

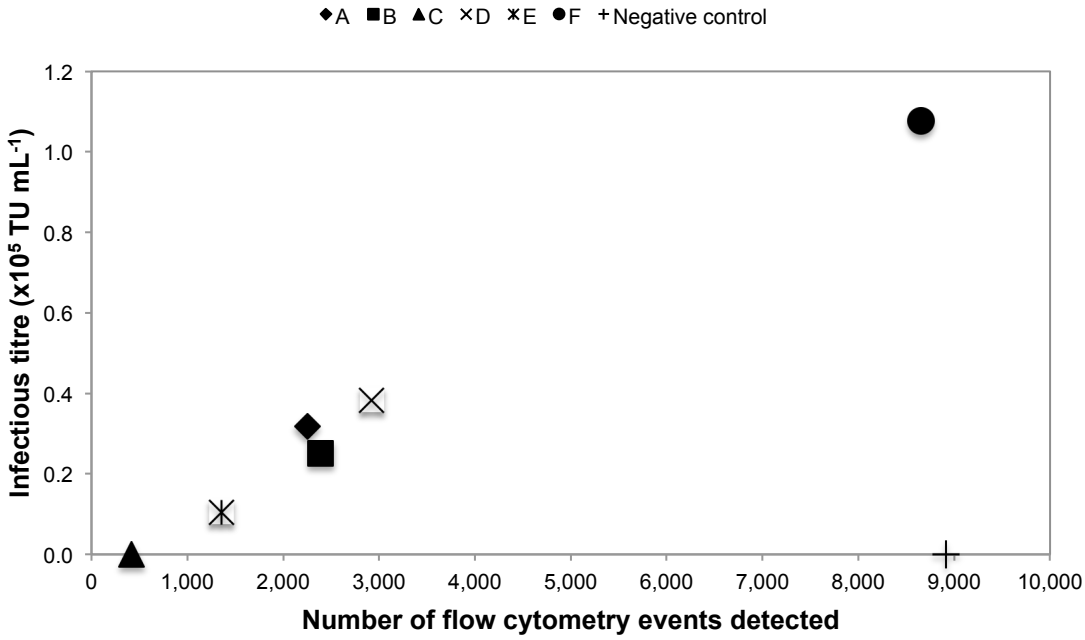


Figure 4.2 Infectious vector titre measured in five lyophilisation buffers (A-F) and a PBS control as a function of the number of flow cytometry events detected following titration on HEK 293FT cells. 50 µL of clarified vector material or PBS (negative control) was added to 250 µL of formulation buffer or PBS, lyophilised, reconstituted in 300 µL of cell culture medium and titrated.

4.3.2.2 Freeze-drying screening study

The model system for this work was a HIV-1-derived LV with an eGFP reporter gene in place of a therapeutic transgene. This LV is pseudotyped with an envelope protein from the retrovirus RD114, which causes it to be more labile than LVs pseudotyped with VSV-G. The half-life of the vector in culture medium at 37°C has been reported to be less than two hours (Strang et al., 2004). This labile virus was chosen intentionally so as to fully challenge the excipient blends evaluated. The results obtained with LV may be applicable to vaccines based on enveloped viruses, such as influenza and enveloped virus-like particles (eVLPs).

Initially, LV samples were lyophilised in the presence of five formulations and a PBS control. Following lyophilisation, a set of samples were exposed to a one-week heat challenge at 37°C, while another set of dried samples were reconstituted and assayed for infectious particle titre. Figure 4.3 (a) shows that vector formulated in just PBS (sample F) is very susceptible to damage by the freeze-drying process and a greater than 90% loss of vector integrity, as measured by infectious titre, was observed. The presence of excipients mitigated the loss of infectious particles with formulations C-E achieving comparable titres pre- and post-lyophilisation. Excipient blends A and B preserved less than 50% of the initial vector titre. These two blends contained a lower concentration of sugars than mixtures C and E, which may be the reason for the reduced level of protection during the lyophilisation process. However, formulation D was simply 0.5 M sucrose and this formulation achieved an average post-lyophilisation titre that was higher than the intial titre. Therefore it is difficult to draw a clear conclusion as to what excipient(s) had a protective effect on the vector infectious titre. Kotani et al. (1994) reported on the freeze-drying of an MLV vector using glucose, sorbitol and gelatin in PBS. Infectious vector recovery was 83% with these excipients, whereas PBS alone only resulted in a 21% recovery. Cruz et al. (2006) screened a range of sugars and proprietary excipients in Tris for long-term storage of lyophilised formulations of a purified MLV vector. The best results were obtained with sucrose and a commercially available excipient called firoin as both led to a preservation of 80% of the pre-lyophilisation titre following freeze-drying. In comparison to both of these studies, the results with excipient blends C-E are very promising.

After the one-week heat challenge, formulation C had the highest average titre, however, this was statistically insignificant (*t*-test, $p = 0.15$) compared to formulation D.

Whilst it may have been beneficial to take both formulations through to an optimisation study, the number of samples for analysis would have been prohibitively large and so formulation C only was taken forward for optimisation (Figure 4.3 (b)).

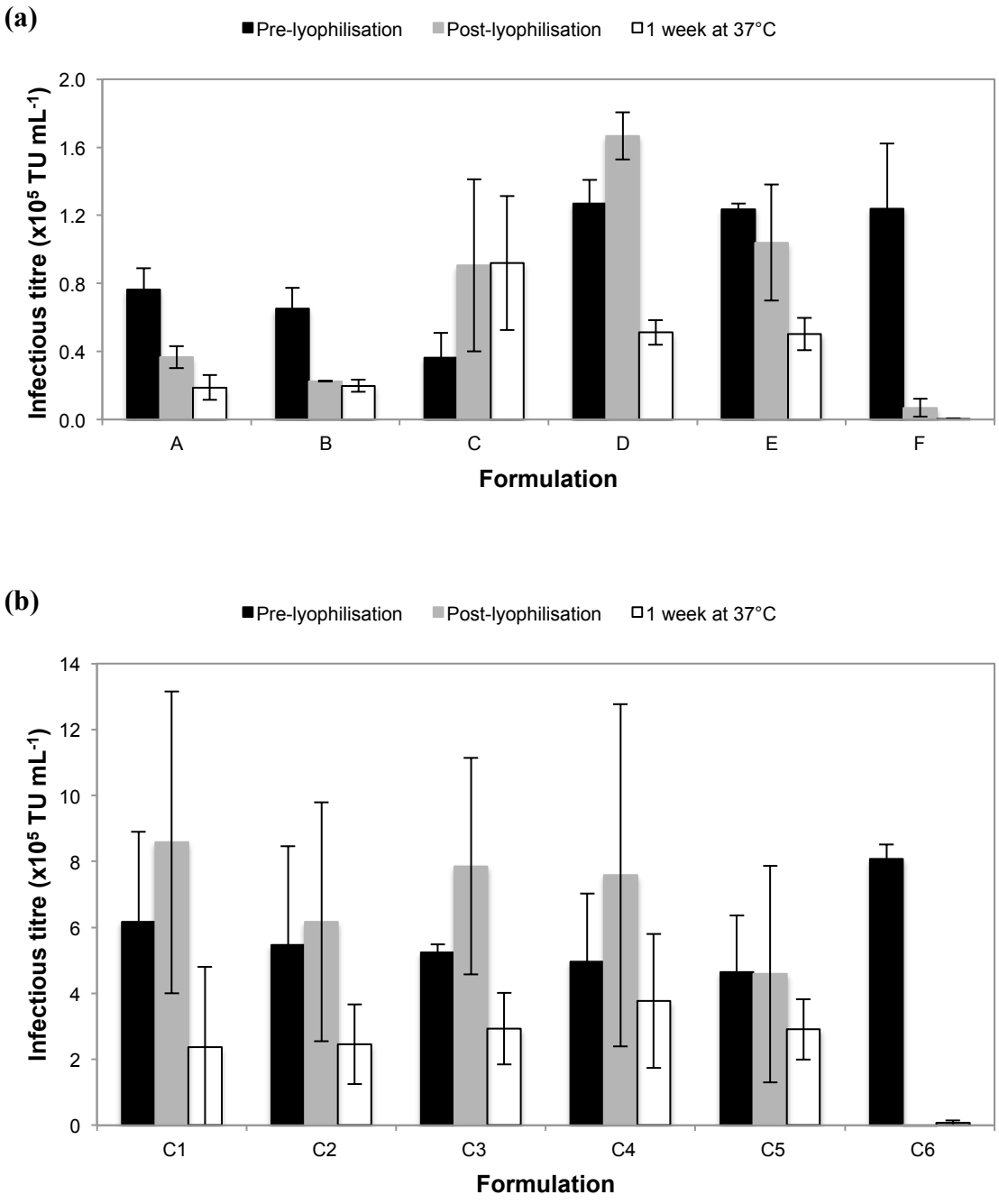


Figure 4.3 The effect of excipient blend on vector integrity following freeze-drying. In (a), five excipient mixes (A-E) are compared to a PBS control (F). Formulation C was subsequently used as the basis for an optimisation run, shown in (b), where five excipient mixes (C1-C5) are compared to a PBS control (C6). Initial pre-lyophilisation titre, post-lyophilisation titre and titre following one week thermochallenge at 37°C are shown in black, grey and white. Error bars represent one standard deviation about the mean (*n* = 3).

4.3.2.3 Freeze-drying optimisation study

Five formulations (C1-C5) based on formulation C were again compared to a PBS control (C6). As before, active vector titres were compared before and after lyophilisation, as well as following heat challenge at 37°C for one week. The results are shown in Figure 4.3 (b). All of the excipient blends provided comparable protection of the vector from the lyophilisation and subsequent reconstitution process with almost no loss in infectious titre. Out of the heat challenged samples, C4 achieved a slightly superior recovery of 76% of the initial infectious LV titre compared to the other formulations. As observed in the initial study, the PBS control (C6) resulted in a negligible recovery of active particles.

4.3.2.4 Freeze-drying followed by long-term storage

To understand if the LV could be stabilised for a longer time period, dried product formulated with C4 was exposed to temperatures of 4°C, 25°C and 37°C for up to three months. A more concentrated virus stock was used for this study and it can be seen in Figure 4.4 (a) that upon freeze-drying, the excipient blend does not maintain the pre-lyophilisation titre as well as it did when a lower vector concentration was used (Figure 4.3 (b)). In a previous study, where similar formulations to the ones used in this study, were used to stabilise adenovirus (Ad5), Stewart et al. (2014) observed a much lower reduction in vector titre following lyophilisation. Ad5 is a nonenveloped virus, and as such does not rely on a fragile envelope protein for host cell infection, thus it can be expected to better retain activity than the labile LV used in this study.

The lyophilised vector titre was measured at 3×10^6 TU mL^{-1}. Dried product, maintained at 4°C for four weeks, contained 84% of recoverable LV when reconstituted and assayed. Following eight weeks, this had dropped to a 65% recovery, at which point the degradation appears to slow down as the same recovery was achieved after 12 weeks. Increasing the temperature to 25°C served to reduce the stability of the vector. While the excipients were able to maintain 68% of the post-lyophilisation titre for up to eight weeks at 25°C, at 12 weeks only 46% of the post-lyophilisation LV titre was recovered. Increasing the temperature to 37°C significantly reduced vector stability and after just four weeks, only 34% of the vector was recovered. Negligible amounts of vector were observed at eight and 12 weeks at 37°C.

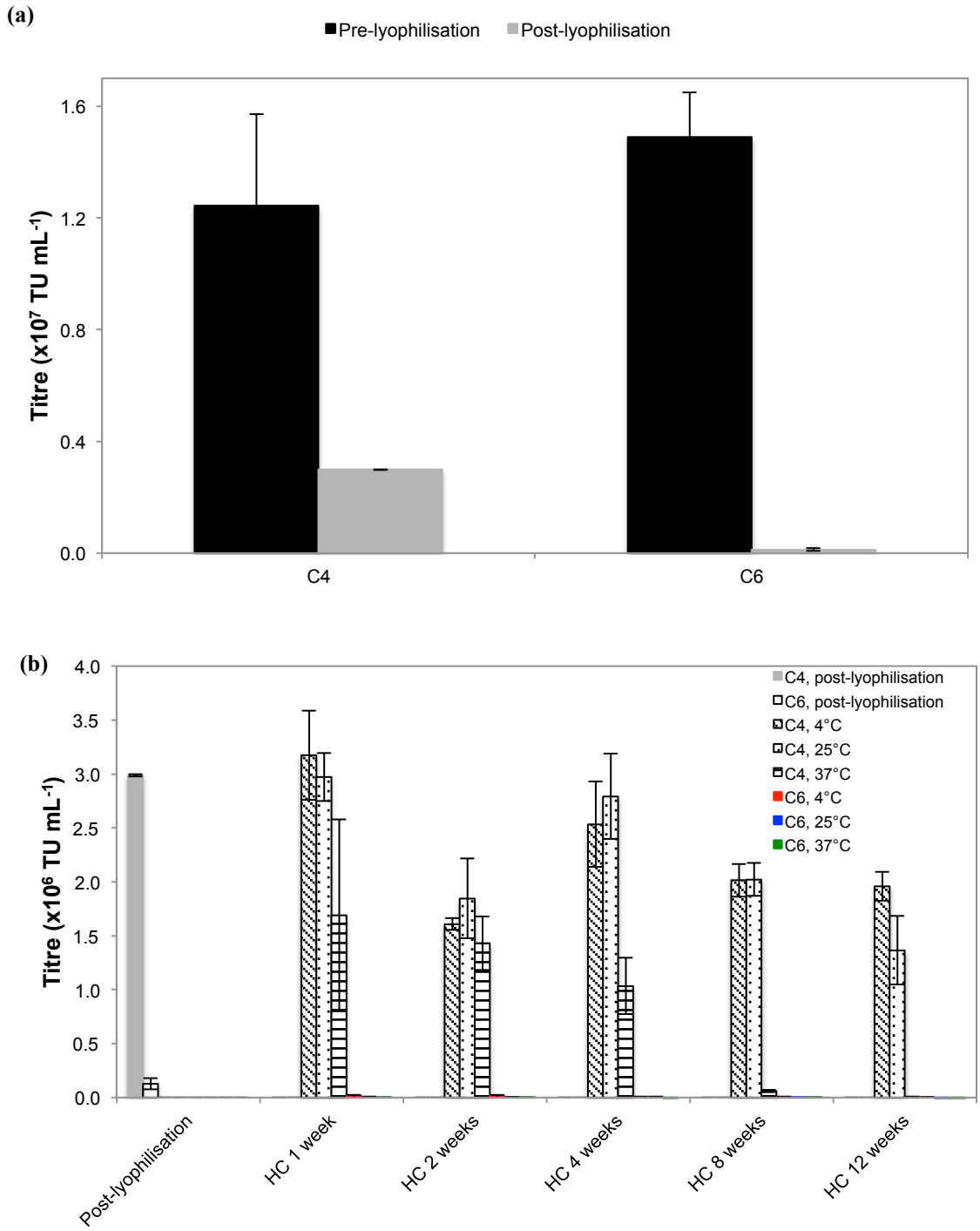


Figure 4.4 The effect on vector infectious titre of freeze-drying in (a) and thermochallenge following freeze-drying at various temperatures and times in (b). An optimised excipient blend (C4) is compared to a PBS control (C6). Formulation C4 achieves comparable activity between 4°C and 25°C, whereas stability is markedly decreased at 37°C. Negligible amounts of infectious particles are observed in C6 following freeze-drying. Error bars represent one standard deviation about the mean (n = 3).

Formulation	Temperature (°C)	Storage time (weeks)	Post-lyophilisation titre remaining (%)
C4	4	1	104 ± 14
C4	4	2	54 ± 1.8
C4	4	4	85 ± 13
C4	4	8	67 ± 5.1
C4	4	12	66 ± 4.5
C4	25	1	99 ± 7.4
C4	25	2	62 ± 12
C4	25	4	93 ± 13
C4	25	8	68 ± 5.1
C4	25	12	46 ± 11
C4	37	1	57 ± 30
C4	37	2	48 ± 8.3
C4	37	4	35 ± 8.7
C4	37	8	2.0 ± 0.4
C4	37	12	0.2 ± 0.1
C6	4	1	10 ± 0.5
C6	4	2	10 ± 0.4
C6	4	4	4.3 ± 0.1
C6	4	8	4.4 ± 0.2
C6	4	12	2.1 ± 0.1
C6	25	1	4.9 ± 0.2
C6	25	2	2.6 ± 0.1
C6	25	4	5.1 ± 0.1
C6	25	8	0.1 ± 0.0
C6	25	12	-0.2 ± 0.0
C6	37	1	0.5 ± 0.0
C6	37	2	0.5 ± 0.0
C6	37	4	-0.2 ± 0.0
C6	37	8	0.1 ± 0.0
C6	37	12	-0.1 ± 0.0

Table 4.6 Percentage of post-lyophilisation infectious titre remaining following storage for 1, 2, 4, 8 and 12 weeks at 4, 25 and 37°C for formulations C4 and C6 (PBS control). Error bars represent one standard deviation about the mean (n = 3).

4.3.3 Study of liquid formulations

4.3.3.1 Vector stability in cell culture supernatant and phosphate buffered saline

In order to have a baseline to compare vector stability improvements in thermostable formulations to, vector stability in cell culture supernatant (CCS) and PBS was determined. Figure 4.5 shows vector stability at 25°C and 37°C for up to 168 h. The vector appears to be more stable in PBS than in CCS. Stability is also improved in 25°C compared to 37°C. The concentration step using a 100 kDa MWCO membrane prior to dilution into PBS will have removed some proteins from the samples. Proteins are known to have a stabilising effect on LVs (Carmo et al., 2008), thus the PBS samples could have been expected to have reduced stability, however, other factors, such as a more optimal pH may contribute to improved stability in PBS.

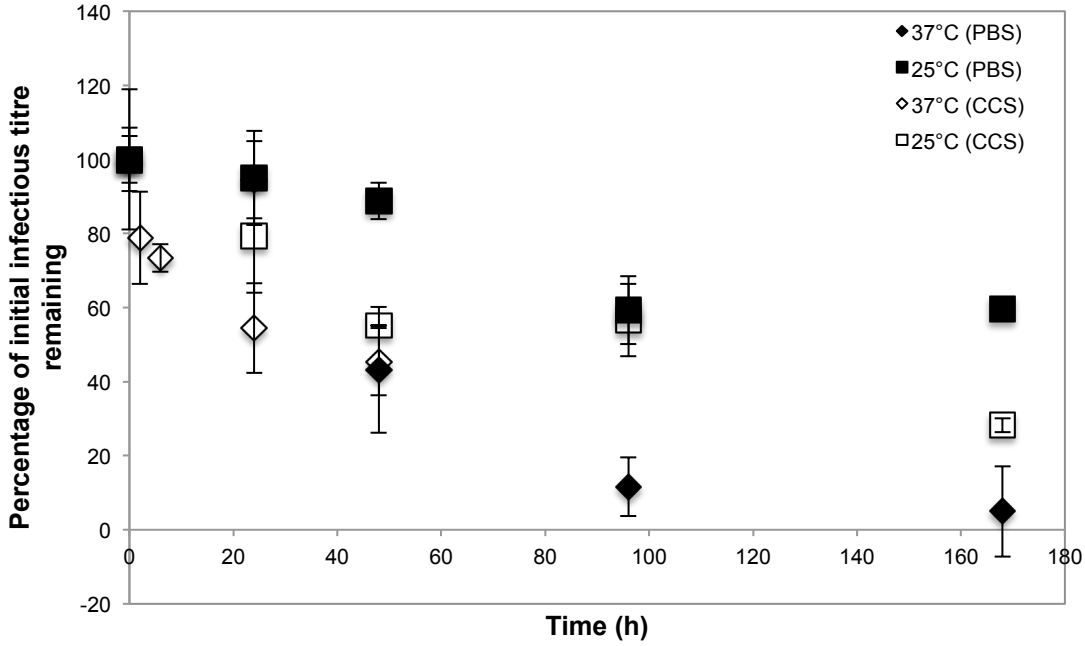


Figure 4.5 Impact of incubation suspending medium, temperature and time on infectious vector titre. Clarified cell culture supernatant (CCS) was either concentrated 10-fold and diluted 10-fold in PBS or aliquoted into cryovials following clarification. Samples were held in a cell culture incubator at either 25°C or 37°C for 168 h (except 37°C CCS samples which were only incubated for 48 h). Error bars represent one standard deviation about the mean (n = 3).

4.3.3.2 Liquid formulation in phosphate-based buffers

For vaccines, it would be convenient if medium-term storage in the liquid state was possible following reconstitution of a freeze-dried formulation. An attempt was therefore made to formulate the LV in a liquid excipient blend. Five formulations (P1-P5) were compared to a PBS control (P6) over a storage period of seven days at 40°C (Figure 4.6). Samples were diluted prior to titration, but the dilution factors were not optimised as for lyophilisation samples. The variability of the t = 0 samples imply that a similar optimisation may have been beneficial. After one day, the titre had been reduced by approximately 80% for all formulations, except the PBS control, for which the titre reduction was less dramatic. Again, this result may have been due to a detrimental effect of the excipient blends on the titration assay cells. After four days, no detectable infectious particles remain for the excipient blends. Excipient blends in Tris-EDTA (TE) buffer were also evaluated, however, no infectious particles were detectable after one day of storage at 40°C.

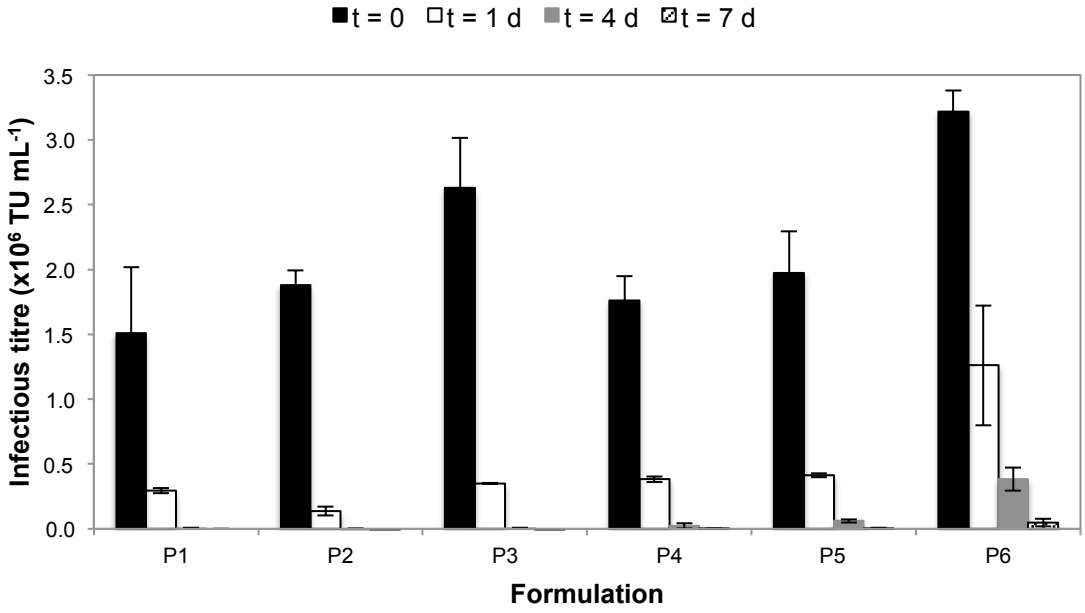


Figure 4.6 Evaluation of phosphate buffer-based liquid formulations for improved LV temperature stability. 50 µL of concentrated CCS was added to 250 µL of formulation. Five phosphate buffer-based formulations (P1-P5) were compared to a PBS control (P6) after incubation at 40°C for 0, 1, 4 and 7 days. Samples were diluted 1:20 with PBS prior to assaying for infectious titre on HEK 293FT cells. Error bars represent one standard deviation about the mean (*n* = 3).

4.4 Conclusion

The infectious titre of this vector decreases by approximately 25% for each freeze/thaw cycle. Formulation in PBS alone resulted in loss of over 90% of infectious LV following freeze-drying. Viral inactivation can be mitigated by the addition of excipient blends, but increased vector concentration reduces the beneficial effects of these blends. An optimised formulation provided protection to the vector for up to 12 weeks at 4°C and eight weeks at 25°C, resulting in recovery rates above 65% compared to the post-lyophilisation titre. The effectiveness of the formulation is reduced in time periods over four weeks at 37°C and is more effective for non-enveloped viruses. Liquid excipient blends based on phosphate and TE buffers were unable to retain infectious titre better than a PBS control.

5. FINAL WORDS AND FUTURE WORK

This section aims to review the thesis objectives, summarise the key findings and provide future recommendations for LV process development.

5.1 Final words

5.1.1 Vector production, harvest and concentration

Vector component production was seen to reduce exponential growth rate and viable cell density for LV packaging and producer cell lines based on HEK 293FT. Suspension adaptation of a packaging cell line was unsuccessful, as cell aggregation could not be alleviated with regular trypsin treatment. The LV titre generated by the stable producer cell line STAR-RDpro was shown to be unaffected by the removal of phenol red from the culture medium, thereby allowing material suitable for AEX purification to be produced. The choice of membrane material for clarification by normal flow filtration was seen to not impact recovery of infectious LV particles. Membrane pore size, however, had a significant impact as a pore size of 0.45 μm led to yields close to 100%, whereas a pore size of 0.22 μm resulted in yields around 40% for all membrane materials. These results were obtained with small filter areas (<9 cm^2), but should scale linearly for maintained feed volume to filter area ratio. Successful vector concentration utilising centrifugal filters was possible with a membrane MWCO of 100 kDa, whereas a 300 kDa MWCO led to low recoveries. The initial flux was significantly higher for the 300 kDa membrane, and it is likely that the resultant fouling was the cause of the low recoveries. For ultrafiltration at larger scales, where there is a means of controlling the initial flux, larger (>100 kDa) pore sizes may enable retention of infectivity.

5.1.2 Capture of an RDpro pseudotyped lentiviral vector using membrane- and monolith-based stationary phases

96-well plates containing monolithic resins with Q and DEAE ligands were found to have reproducible protein binding capacities and were chosen for initial development of an AEX capture step. LV binding was studied in the plate format and found to be successful in all conditions except for when vector was loaded in phosphate buffer. Subsequently salt concentration for elution was evaluated for Q and DEAE monoliths and also Q membrane, all in the plate format. Immediate dilution of the eluates was

found to be necessary to avoid LV inactivation. A NaCl concentration of approximately 1 M was required for elution of meaningful LV quantities for all three stationary phases. This result, however, was not replicated when gradient elution was used for 1 mL versions of the Q monolith and Q membrane. In this format, LV eluted at approximately 0.4 M NaCl and the recoveries were significantly lower.

5.1.3 Formulation of an RDpro pseudotyped HIV-1-derived lentiviral vector

LV titre was found to decrease by approximately a quarter for each freeze/thaw cycle. Freeze-drying of LV in PBS led to loss of over 90% of functional titre whereas freeze-drying in excipient blends enabled insignificant change in titre. Storage for up to 12 weeks at 4°C and up to eight weeks at 25°C with more than 65% retention of post-lyophilisation titre was possible for an optimised excipient blend. Liquid excipient blends based on phosphate and TE buffers were unable to retain infectious titre better than a PBS control during thermochallenge at 40°C.

5.2 Future work

LV bioprocessing is still in its infancy and the work presented in this thesis raises more questions than it provides answers. The most commercially interesting applications of LVs are for cancer immunotherapy and given that autologous cell processing is likely to remain costly, there will be pressure to reduce treatment COGs by producing large vector batches, thereby benefiting from economies of scale such as reduced expenditure on quality control. Future LV process development work therefore needs to focus on economical and scalable technologies.

5.2.1 Cell culture

Future work for upstream processing of LVs should focus on optimisation of transient transfection protocols for suspension cells and the generation of high titre, stable producer suspension cell lines. The use of suspension cells should lead to robust, scaleable processes as it allows the use of controllable bioreactors and volumetric scale-up. Important factors to optimise for transient transfection protocols include choice of cell line, transfection reagent, pDNA to transfection reagent ratio, culture medium composition, bioreactor operation and harvest strategy. For the generation of stable producer cell lines, approaches that are not based on sequential introduction of the

packaging and transfer vectors into random locations in the host cell genome would be interesting to explore. Targeted approaches that allow introduction of the vector components on one construct (e.g., Cirmirakis, 2014) may result in improved titres. Vector safety is maintained by separating expression at the mRNA level.

In cases where suspension adaptation of stable producer cell lines is the preferred option, clonal dilution in 96-well plates and screening for non-aggregating, high producer cells is likely to offer a better outcome than the approach taken in Chapter 2 where a small number of shake flasks were used. If not already present in the culture medium, the addition of a sulphated polyanion such as dextran sulphate may be beneficial to reduce cell aggregation (e.g., Zanghi et al., 2000).

5.2.2 Primary recovery

The normal flow filtration protocols with a series of filters of decreasing pore size adapted for production of LV for clinical trials is viable for commercial supply of vector where adherent cell culture is feasible (i.e., for the treatment of very rare diseases, such as ADA-SCID and WAS). However, for the treatment of conditions with larger patient populations, such as β-thalassemia and blood cancers, where upstream processing needs to be based on suspension culture in bioreactors, an alternative primary recovery strategy is needed as the solids content of the harvest is dramatically increased compared to adherent culture harvests. Large-scale CHO cell culture material is typically harvested with disc-stack centrifugation followed by depth filtration and 0.22 μm membrane filtration (Liu et al., 2010), whereas for small to medium scale, disposable depth filtration followed by 0.22 μm membrane filtration is the preferred option (Pegel et al., 2011). The vast majority of commercially available depth filters are positively charged, thereby providing DNA removal. The positive charge means that depth filters bind the negatively charged LVs. Primary recovery strategies developed for recombinant protein and mAb processes are therefore of limited use to LV process developers. The evaluation of alternative harvest methods, such as tangential flow microfiltration and dynamic body feed filtration, are of interest for LV processing. Direct capture methods, combining harvest and purification, may also be beneficial for LV bioprocessing as reduced handling and processing time is likely to lead to improved overall product recovery. The unfashionable method expanded bed adsorption (EBA) could therefore be an option, although vector recoveries are likely to be low with non-

specific methods such as AEX. EBA resins may also need modernisation to bring binding capacities up to the level of contemporary AEX stationary phases. Methods based on porous membranes with sufficient pore size to allow cells and cell debris to pass through the membrane, while binding vector, could be a modern alternative to EBA (Hardick et al., 2013). Commercially available Direct Capture technology from Sterogene Bioseparations is based on a packed bed of large (compared to traditional packed beds) beads that result in interstitial spaces of sufficient size to allow cells and cell debris to pass through the bed, while product is retained. Again, this would be an interesting technology to evaluate for LV processing.

5.2.3 Purification

As described above, the choice of primary recovery method is heavily impacted by whether adherent or suspension cells are used for LV expression. Upstream processing choices also have an impact on purification. Adherent culture for LV production is normally carried out with 10% FBS, whereas suspension cells typically grow in serum-free medium, thereby dramatically reducing the burden on purification steps. Purification is also influenced by whether transient transfection or a stable producer cell line is used for expression as transient transfection involves the addition of large amounts of pDNA. For purification of LV generated by a stable producer cell line grown in serum-free medium, where protein and DNA impurity level is minimised, it would be interesting to evaluate GE Healthcare's Capto Core 700 resin. This resin was developed for flow-through purification of viruses, thereby avoiding the difficulties associated with LV elution from bind-and-elute resins.

In Chapter 3, promising results were obtained with a membrane and a monolith with Q ligands in 96-well plates, however, the results were not repeatable in 1 mL versions of the supports. Poor resolution was obtained with the Q membrane, which may be improved in a new version of the Sartobind capsules from Sartorius where bed height has been increased and the void volume subsequently reduced. Once bound to a Q support, LV appears very difficult to elute and this is the likely reason for the fields' preference for DEAE exchangers. As shown for baculovirus purification, it may be beneficial to reduce the ligand density of AEX stationary phases (Vicente et al., 2011). Although likely to require more optimisation than AEX resins, it may be worthwhile to explore mixed-mode chromatography supports for LV purification.

To date, affinity purification of LVs has had limited success (Cheeks et al., 2009; Segura et al., 2007). Although likely to be troublesome from a regulatory perspective, evaluation of the use of self-cleaving intein-based purification tags, as described for proteins expressed in *Escherichia coli* (*E. coli*) (Mitchell and Lorsch, 2015), could be interesting for LVs not already in clinical development. The real step change in LV purification is likely to occur when a cost-effective affinity resin that allows for gentle elution conditions becomes available. In terms of already commercially available affinity ligands, as Env are glycoproteins, LVs may bind to a lectin affinity resin such as GE Healthcare's Capto Lentil Lectin. Although not seemingly cost-effective (£1,738 for 100 mL of resin (GE Healthcare, 2015)), elution is carried out by addition of carbohydrates, which may allow for good retention of infectious particles.

5.2.4 Formulation

The freeze-dried formulations evaluated in Chapter 4 showed promise for long-term storage of LV. The need for large dilutions of the freeze-dried material before titration led to the introduction of large variability, thus some further development of the titration protocol may be useful. If considering formulation of LV, rather than formulation of vaccines for use in the developing world, it would be interesting to look at long-term storage at -20°C to 4°C. Energy consumption and logistical complexity is reduced if the need for <-65°C storage can be avoided.

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6. APPENDIX 1 – T-TEST CALCULATIONS

Statistical significance was tested for using the the t-test formula (Equation 6.1):

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \tag{6.1}$$

where t is the t-test value, $\bar{x}_1$ is the mean of the first set of values, $\bar{x}_2$ is the mean of the second set of values, s_1 is the standard deviation of the first set of values, s_2 is the standard deviation of the second set of values, n_1 is the total number of values in the first set of values and n_2 is the total number of values in the second set of values.

Standard deviations were calculated using Equation 6.2:

$$s = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(x_i - \bar{x})^2} \tag{6.2}$$

where s is the standard deviation, n is the total number of values for each set of replicates, x_i is the individual values and $\bar{x}$ is the mean of the values for each set of replicates.

Table 6.1 contains a sample calculation for t-test values presented in Section 2.3.5, using a two-tailed test and assuming equal variance for the means.

	0.45 µm membrane pore size	0.22 µm membrane pore size
Replicate 1	97	41
Replicate 2	99	43
Replicate 3	109	40
Replicate 4	100	36
Replicate 5	107	45
Replicate 6	117	43

$$s_1 = \sqrt{\frac{1}{6}\sum_{i=1}^{6}(x_i - 105\%)^2} = 7.6\%$$

$$s_2 = \sqrt{\frac{1}{6}\sum_{i=1}^{6}(x_i - 41\%)^2} = 3.1\%$$

$$t = \frac{105\% - 41\%}{\sqrt{\frac{7.6\%^2}{6} + \frac{3.1\%^2}{6}}} = 19$$

Degrees of freedom = $n_1 + n_2 - 2 = 6 + 6 - 2 = 10$

Using 10 degrees of freedom, a t-table can be used to compare the calculated t-value to that required for significance at the different probability values. In this case, the t-value should be larger than 2.23 for a probability of p = 0.05, 3.17 for p = 0.01 and 4.59 for p = 0.001. The result is highly significant.

Using a computational tool the p-value can be calculated as 3.8×10^{-9}.

Table 6.1 Sample t-test calculation for experiment assessing the impact of clarification membrane pore size.

Thesis section	*t*-value	*p*-value	Degrees of freedom
2.3.4 (culture medium comparison)	1.5	0.20	4
2.3.5 (membrane material comparison) (Please note that three calculations had to be performed as a *t*-test can only be used to compare two treatments at a time, whereas this experiment looked at three different membrane materials.)	1.4 (regenerated cellulose compared to PES); 2.8 (regenerated cellulose compared to PVDF); 1.2 (PVDF compared to PES)	0.30 (regenerated cellulose compared to PES); 0.11 (regenerated cellulose compared to PVDF); 0.36 (PVDF compared to PES)	1
2.3.5 (membrane pore size comparison)	19	3.8×10^{-9}	10
2.3.5 (syringe filter compared to Stericup)	6.8	0.02	1
4.3.2.2 (formulation C compared to formulation D)	1.8	0.15	4

Table 6.2 Summary of *t*- and *p*-values used in thesis.

In this thesis results with p-values < 0.05 have been considered significant.